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MINIREVIEWS

Renal progenitors: Roles in kidney disease and regeneration

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Abstract

Kidney disease is a devastating condition that affects millions of people worldwide, and its prevalence is predicted to significantly increase. The kidney is a complex organ encompassing many diverse cell types organized in a elaborate tissue architecture, making regeneration a challenging feat. In recent years, there has been a surge in the field of stem cell research to develop regenerative therapies for various organ systems. Here, we review some recent progressions in characterizing the role of renal progenitors in development, regeneration, and kidney disease in mammals. We also discuss how the zebrafish provides a unique experimental animal model that can provide a greater molecular and genetic understanding of renal progenitors, which may contribute to the development of potential regenerative therapies for human renal afflictions.

Key words: Kidney; Renal progenitor; Nephrogenesis; Development; Nephron; Regeneration; Zebrafish; Parietal epithelial cell; Tubular progenitor cell

Core tip: The kidney is a complex organ comprised of many diverse cell types. Damage of renal cells leads to devastating kidney diseases because humans have limited abilities to regenerate these cells. Here, we explore recent research that has sought to better characterize renal progenitors during development, to identify whether renal stem cells exist in the adult kidney, and to understand the enigmatic properties of renal progenitors across diverse vertebrate species such as fish.

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INTRODUCTION

The kidney is a vital organ comprised of many specific

cell types that work in conjunction to maintain body fluid homeostasis[1]. Notably, this organ is responsible for regulating pH, secreting hormones, maintaining blood pressure, and controlling red blood cell numbers[2]. Each human kidney contains up to 2 million functional units called nephrons that are divided into distinct epithelial segments[3]. Nephrons are organized within an intricate tissue architecture, where they are joined to a centralized collecting duct (CD) network for waste excretion[4]. Due to the complexity of the kidney, the coordination of developmental events that create nephrons and their surrounding interstitial populations from embryonic progenitor cells remains a key question in the biomedical field.

Previous studies using the murine animal model have shown that the mammalian kidney is derived from $Osr1^+$ cells of the intermediate mesoderm (IM) (Figure 1)[5]. The $Osr1^+$ cells give rise to the metanephric mesenchyme (MM), which condenses to form the cap mesenchyme (CM) (Figure 1)[6]. The CM is a self-renewing renal stem cell population from which nephrons are crafted through a reiterative, coordinated process that involves inducing cohorts of CM cells to undergo a simultaneous mesenchymal-to-epithelial transition (MET) upon receiving differentiation signals from the adjacent ureteric bud (UB) (Figure 1)[7]. A pre-tubular aggregate arises from each cohort of these induced renal progenitors, which ultimately becomes an epithelialized renal vesicle (RV) (Figure 1). The activated RVs signal reciprocally to the UB to undergo branching morphogenesis, eventually forming an elaborate, arborized CD network[8]. Meanwhile, the RV structures undergo proliferation and morphogenesis, changing to form a comma-shaped body (CSB) followed by an S-shaped body (SSB) (Figure 1)[9]. The SSBs undergo further elongation and maturation, becoming an intricately segmented nephron (N) structure that connects to the CD system (Figure 1), and contains discrete glomerular, proximal, and distal regions[10].

During development, nephrogenesis involves a synchronized sequence of dynamic cellular events reliant upon the replenishment of the self-renewing CM and the subsequent patterning of the renal progenitors. Interestingly, nephrogenesis in mammalian and avian species ceases either at the end of gestation or shortly after birth, while in other vertebrates such as fish, reptiles, and amphibians, nephrogenesis has been documented to occur throughout the animal's lifetime[11,12]. Further, the mammalian kidney is believed to be an organ with relatively limited regenerative potential compared to structures such as the liver[2]. This is problematic, as kidney disease is an escalating global health issue in today's society. Upon acute injury, however, the mammalian kidney has been observed to undergo considerable structural remodeling and repair[13-15]. Whether an endogenous adult stem cell population contributes to this process of epithelial regeneration remains controversial.

RENAL PROGENITORS DURING MAMMALIAN DEVELOPMENT

In 2008, Kobayashi *et al*[16] provided the first evidence of multipotent renal progenitors in the developing mammalian kidney. The investigators' approach consisted of using transgenic mice to perform lineage tracing of $Six2^+$ cells. It had been previously shown that the transcription factor Six2 is necessary for nephrogenesis during murine development[17]. During early stages of nephron induction, $Six2^+$ labeled cells were observed in the CM surrounding the UB epithelium[16]. The CM progenitors receive signals from the UB to either self-renew, thus exhibiting a key stem cell attribute, or undergo MET and differentiate into distinct epithelial segments of the nephron[6]. Under the correct signals, these $Six2^+$ cells form RV, and subsequently the SSB. This RV progenitor pool eventually gives rise to multiple epithelial cell types that comprise the nephron including proximal tubular cells, distal tubular cells, connecting tubular cells, and podocytes. Interestingly, the $Six2^+$ progenitors did not contribute to the CD, vasculature, or interstitium. The transcriptional regulator, Osr1, is broadly expressed in the IM, and was found to be required for the formation of the $Six2^+$ progenitor population[5]. In a separate study by Boyle *et al*[18] (2008) a transgenic strategy was employed to trace a renal progenitor pool expressing Cited1. Similar to the previously mentioned study[16], the $Cited1^+$ progenitors are induced in the MM and continually contribute to nephron formation during kidney organogenesis. Over time, the self-renewing CM stem cells cease to self-renew and found a final wave of nephrons at the cortex of the metanephros[9].

In the following sections, we discuss how the maintenance of renal stem or progenitor cells in the adult kidney has been debated extensively based on a series of conflicting experimental observations. The existence of renal stem/progenitor cells has been proposed as an explanation for the observation that injuries to nephron epithelial cells can be healed through replenishment with newly proliferative cells (Figure 2). At present, however, it remains an unsettled controversy as to whether the adult mammalian kidney contains self-renewing renal progenitors or can be induced to form cells that exhibit stem cell-like behaviors in the context of renal injuries and other disease conditions.

EARLY EVIDENCE FOR ADULT RENAL STEM CELLS IN MAMMALS

In 2003, Maeshima *et al*[19] identified progenitor-like cells scattered throughout the tubules of the adult rat kidney by utilizing *in vivo* BrdU labeling. Upon ischemic injury, the label-retaining cells underwent multiple cell divisions, becoming positive for proliferating cell nuclear antigen[19]. The progeny of the $BrdU^+$ cells first expressed vimentin,

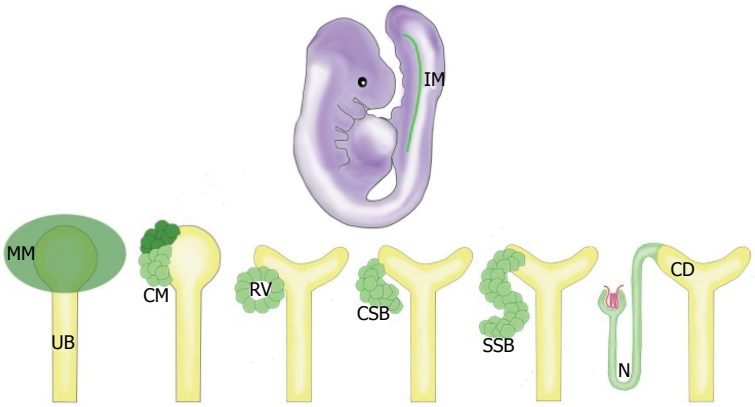

Figure 1 The progression of kidney organogenesis in mammals. The MM is derived from the IM. The MM condenses to form the CM, a renal progenitor population. These progenitors receive signals to self renew (dark green) or differentiate (light green). Cells receiving differentiation signals are organized into an epithelialized RV. Upon further maturation, these cells form a CSB, then an SSB, and finally the N. IM: Intermediate mesoderm; MM: Metanephric mesenchyme; UB: Ureteric bud; CM: Cap mesenchyme; RV: Renal vesicle; CSB: Comma-shaped body; SSB: S-shaped body; N: Nephron; CD: Collecting duct.

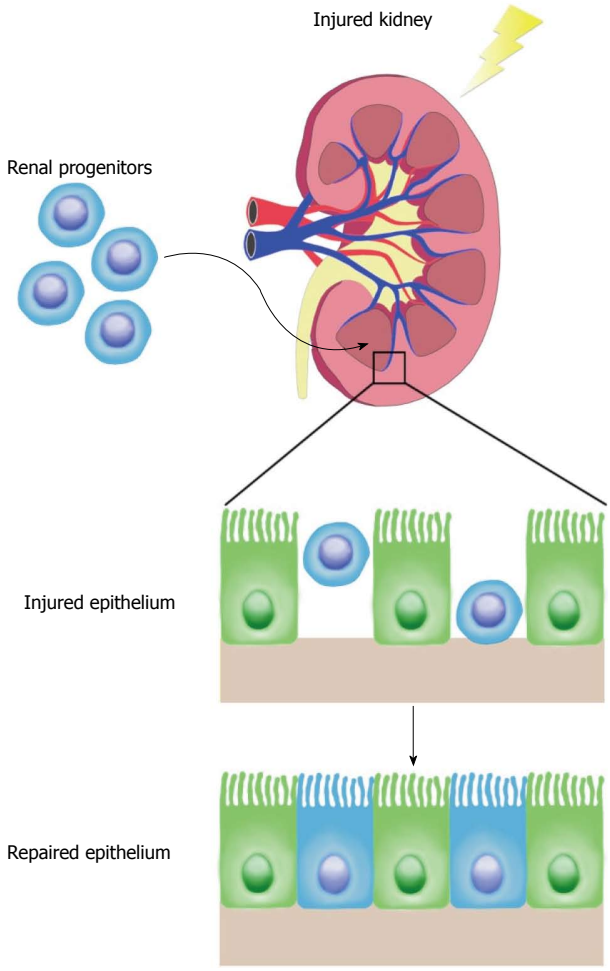

Figure 2 Regenerative capacity of adult renal progenitors. Proposed adult renal stem cells were isolated and transplanted into hosts with injured kidneys. These progenitors homed to the site of injury and repopulated the renal tubular epithelium robustly. This supports a model in which an adult stem cell population contributes to kidney regeneration after acute injury.

a mesenchymal marker, but later began to express E-cadherin, an epithelial cell marker[19]. Collectively, results from this study suggest that label-retaining cells are renal progenitors that contribute to regeneration of the rat kidney.

In a follow-up study, Kitamura *et al*[20] (2005) dissected a single nephron from an adult rat kidney and isolated a cell line (rKS56) with high proliferative potential. Upon genetic analysis, the rKS56 cells expressed both developmental markers and mature tubular markers[20]. When these cells were transplanted into injured rat kidneys, they readily engrafted, restored tubules, and improved renal function[20]. These rat studies support the existence of renal adult stem cells that possess the capability to repair tissue and self-renew.

In the same year, Bussolati *et al*[21] (2005) discovered $CD133^+$ progenitor cells derived from the adult human kidney. These cells expressed Pax2, which is an embryonic kidney marker, and were capable of expansion and self-renewal *in vitro*[21]. Interestingly, when these cells were implanted subcutaneously into immunocompromised mice, they formed tubules expressing renal epithelial markers[21]. Upon intravenous injection of $CD133^+$ cells into mice with acute tubular injury, they homed to the kidney and assimilated into the proximal and distal tubules[21]. These data support that an adult stem cell population exists in the adult kidney and may participate in regeneration after injury.

In 2006, Dekel *et al*[22] isolated $Sca1^+Lin^-$ multipotent progenitors that were distinct from hematopoietic stem cells from the adult mouse kidney by fluorescence-activated cell sorting. Upon transplantation of this population into mice with ischemic injured kidneys, the cells engrafted into the interstitial space and repopulated the renal tubule[22]. Because the $Sca1^+Lin^-$ progenitors were able to contribute to tubule repair, this provides further evidence that may suggest the existence of resident adult renal stem/progenitor cells in mammals.

PARIETAL EPITHELIAL CELLS AS RENAL PROGENITORS

Previous findings suggest that renal progenitor cells (RPCs) are present in humans and may be the origin of podocyte replacement (Figure 3)[21,23,24]. In humans, these RPCs are a subset of parietal epithelial cells (PECs) located in Bowman's capsule that coexpress species-specific surface markers CD133 and CD24. Under correct culture conditions, $CD133^+CD24^+$ PECs have the potential to differentiate into podocytes or tubular epithelium[24]. However, in some cases activation of RPCs can be harmful,

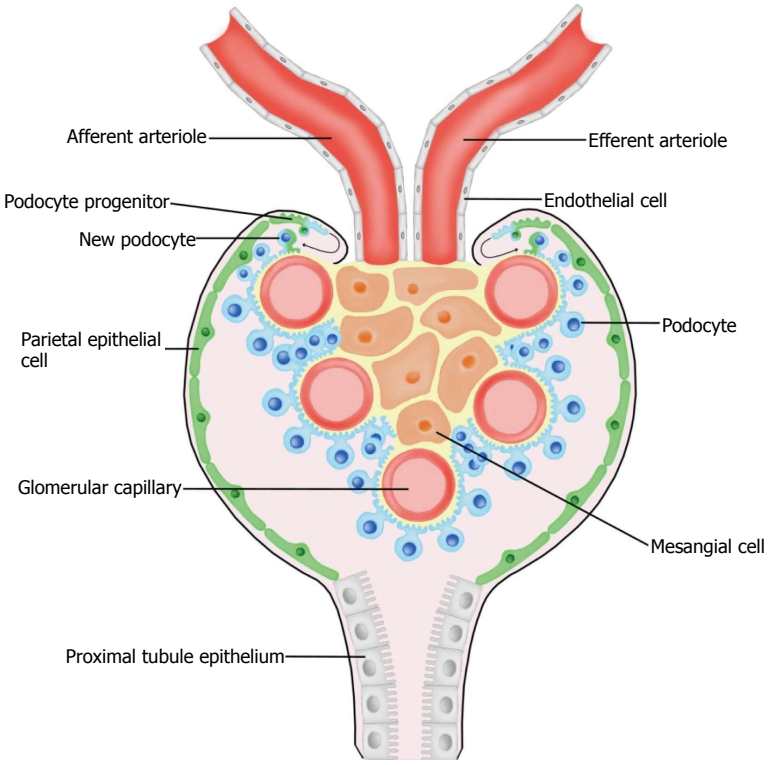

Figure 3 A model of podocyte maintenance and regeneration. Parietal epithelial cells (green) line the inside of Bowman's capsule, and are suggested to serve as a renal progenitor population. Upon acute injury, parietal epithelial cells have been observed to give rise to fully differentiated podocytes (blue).

as they have been shown to contribute to hyperplastic lesions within the glomerulus leading to degenerative disease[25].

Wanner *et al*[26] (2014) investigated the regenerative role of RPCs during glomerular aging and injury. The researchers further characterized the function of PECs during kidney development by using a transgenic mouse system where upon administration of doxycycline, PECs become genetically labeled with membrane-tagged green fluorescent protein. Upon further analysis, mice exposed to doxycycline from days E8.5 to P28 exhibited mG-labeled cells with foot processes, indicating that PECs can give rise to fully differentiated podocytes. Then, the researchers induced acute podocyte loss in an mT/mG reporter strain of mice by utilizing an inducible diptheria toxin receptor system. In this context, only podocytes coexpressing mG and the diptheria toxin receptor are ablated. Upon flow cytometric analysis of kidneys four weeks after ablation, it was observed that there was a significant increase in the numbers of resident mT-labeled podocytes. This data illustrates how podocytes possess regenerative capacity after acute injury. Alternatively, in a unilateral nephrectomy damage context, podocyte turnover was not detected. In addition, it was observed that during aging, podocyte renewal does not occur. Taken together, these results suggest that podocyte regeneration seems to be limited to developmental and acute injury contexts. This study was the first to report that PECs can form fully differentiated podocytes, however their model does not identify the source of the new podocyte population after acute injury.

In a recent study conducted by Lasagni *et al*[27] (2015) the regenerative potential of these RPCs in response to podocyte injury was studied in mice. In order to examine if the generation of new podocytes influences disease outcome, an inducible transgenic mouse model (NPHS2.iCreER^{T2}; mT/mG) was used. Upon tamoxifen administration, podocytes were genetically labeled with GFP, while all other kidney cells were labeled with TomRed. Although, after tamoxifen withdrawal, newly generated podocytes are labeled with TomRed. Mice were injected with doxorubicin to induce Adriamycin nephropathy and later biopsied, where the numbers of GFP$^+$/Syn$^+$ cells (pre-existing podocytes) and TomRed$^+$/Syn$^+$ cells (newly generated podocytes) were counted. It was found that a significant increase of newly generated podocytes occurred after injury. In addition, it was determined that remission of proteinuria in these mice is associated with the generation of new podocytes. These data suggest that RPCs may play a role in the remission of glomerular disease in mice.

Further, a model for RPC lineage tracing was established by Lasagni *et al*[27] (2015) using an inducible transgenic mouse line where green, yellow, cyan, or red are randomly expressed under the control of the Pax2 promoter. It was observed that Pax2$^+$ cells localized in the parietal epithelium of the glomerulus are progenitors that give rise to podocytes during postnatal kidney development. Interestingly, nascent podocytes were labeled with different colors, indicating that these cells did not arise due to clonal division of a single progenitor. These Pax2$^+$ RPCs were found to be responsible for podocyte regeneration in the Adriamycin nephropathy disease context. Mice with proteinuria remission exhibited abundant intraglomerular Pax2$^+$ cells surrounding capillaries. Conversely, mice with persistent

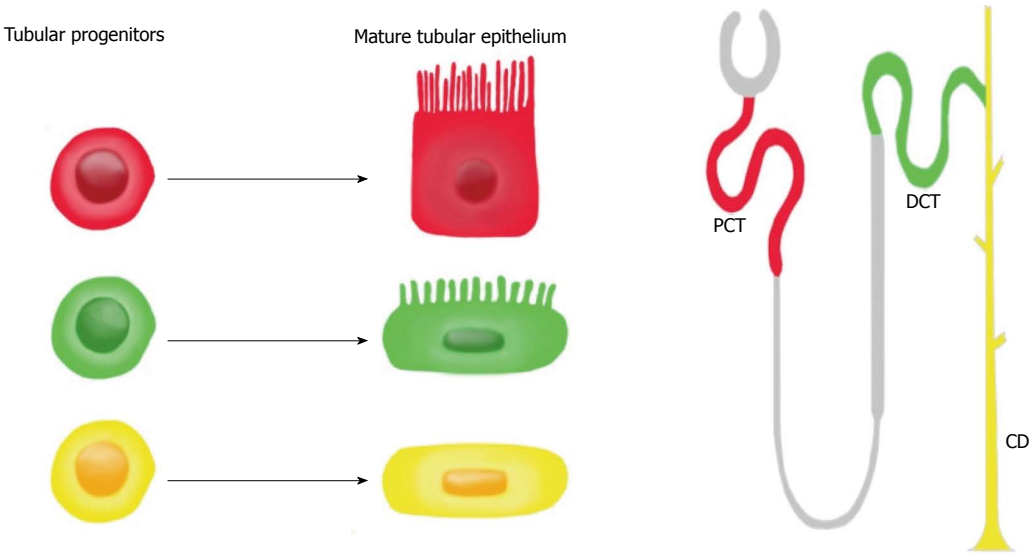

Figure 4 The fate of tubular progenitors. Tubular progenitors are believed to be involved in renal tissue maintenance and regeneration. This stem cell population is lineage-restricted to a specific segment of the nephron. For example, the red progenitor is predestined to become PCT, the green progenitor is predestined to become DCT, and the yellow progenitor is predestined to become CD. PCT: Proximal convoluted tubule; DCT: Distal convoluted tubule; CD: Collecting duct.

proteinuria exhibited virtually no intraglomerular $Pax2^+$ cells. Furthermore, treating diseased animals with the GSK3 inhibitor BIO significantly increased the number of $Pax2^+Syn^+$ cells. All eight BIO-treated mice underwent proteinuria remission, where only two DMSO-treated mice exhibited proteinuria remission. Significantly, the differentiation of RPCs into podocytes can be pharmacologically driven in order to reverse glomerular disease.

RESIDENT TUBULAR PROGENITOR CELLS

In 2011, Lindgren *et al*[28] provided the first evidence for the existence of tubular progenitor cells in humans (Figure 4). In this study, progenitor cells were isolated from renal tissue by cell sorting for high ALDH enzymatic activity. It was observed that these progenitors were scattered throughout the proximal tubules and displayed stem cell properties such as sphere formation and anchorage-independent growth[28]. Human tubular progenitors are localized in the proximal tubule and distal convoluted tubule and possess the following expression profile: $CD133^+CD24^+CD106^-$. Upon injection of these progenitors into SCID mice with acute kidney injury, these cells were able to engraft, form new tubule cells, and improve renal function[29].

Recently, Rinkevich *et al*[30] (2014) sought to further characterize renal tubular progenitor involvement in development, maintenance, and regeneration. The investigators crossed Actin CrER mice with "Rainbow" mice in order to genetically trace individual epithelial cells within the adult kidney. Offspring were injected with tamoxifen at 12 wk old, and were sacrificed at varying time points for clonal analysis. After 1 mo, 2-3 cell clones were scattered throughout the renal cortex, medulla, and papillae. These singly colored clones later grew in size and contributed to existing tubules. The composition of these clones was further examined by immunostaining for segment-specific markers, where it was determined that they did not expand into different segments. These results suggest a model in which tissue-restricted progenitor cells are responsible for kidney maintenance.

In addition, the researchers performed similar clonal analysis during kidney development, where they traced embryonic renal progenitors from E13.5 to P1[30]. Resulting tubules were observed to be polyclonal, indicating several progenitor cells are present during organogenesis. Immunostaining for segment-specific markers revealed clones separately composed of proximal tubule, distal tubule, or CD fates. These results support that renal progenitors during development are lineage-restricted to a specific tubule type. Furthermore, the clonal response to acute injury was studied by performing unilateral ischemia/reperfusion to the left kidneys of adult animals. After 2 mo, single colored clones appeared restricted to specific tubule segments. In damaged areas, significant tubule regeneration was observed where clones contributed circumferentially to the entire tubule. The clones expanded longitudinally and perpendicularly within the same tubule, however they did not extend into adjacent segments of the nephron or invade into neighboring nephrons. Upon long-term fate analysis, clones maintained the identity of a single epithelial lineage. These adult renal clones were found to originate from Wnt-responsive precursors that form segment specific tubules. Harvested kidneys from transgenic animals were dissociated into single cells and cultured in Matrigel to form organoids. Each monoclonal renal sphere was comprised of a distinct epithelial cell type. Collectively, data from this study supports the existence of fate-restricted progenitors that function in

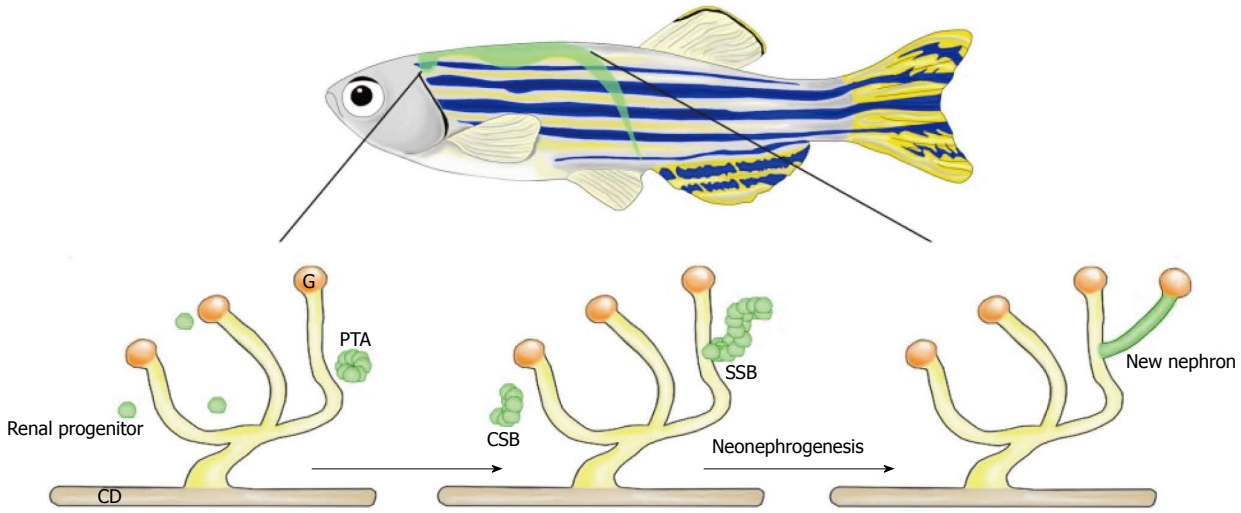

Figure 5 Neonephrogenesis in the adult zebrafish. Adult zebrafish possess the unique ability to generate new nephrons during adulthood. Neonephrogenesis in zebrafish mimics the cellular dynamics of nephrogenesis during mammalian kidney development. Renal progenitors cluster to create a PTA. This aggregate changes morphology as it first forms a comma-shape followed by an S-shape. The SSB differentiates into specific cell types that comprise the blood filter and tubules of a mature nephron. CD: Collecting duct; G: Glomerulus; PTA: Pre-tubular aggregate; CSB: Comma-shaped body; SSB: S-shaped body.

maintaining and regenerating the mammalian kidney[30].

FISH AS A MODEL TO STUDY RENAL PROGENITORS AND REGENERATION

Renal progenitors exist in the adult kidney across many different vertebrate species, such as fish (Figure 5)[11]. In lower vertebrates, renal regeneration and structural remodeling occurs in response to injury due to the presence of potent renal progenitors. Interestingly, the presence of these progenitors can result in the formation of new nephrons during adult growth as well as during regeneration, in a process termed neonephrogenesis[4]. In stark contrast, mammals cease the generation of new nephrons at birth or shortly after[9]. While we have previously discussed observations that have led the hypothesis that renal progenitors may exist in the adult mammalian kidney, there are alternative views including the generation of scattered progenitors in response to injury[31]. Despite such controversies, it is well accepted that the mammalian kidney responds to resection with compensatory glomerular and tubular hypertrophy[32].

To date, the existence of renal progenitors capable of neonephrogenesis has been most extensively documented in a number of fish species including skate, zebrafish, dogfish, rainbow trout, catfish, goldfish, toadfish, and tilapia[33-44]. A deeper understanding of the molecular mechanisms driving neonephrogenesis in these fish may provide novel insights in the pathogenesis of human kidney diseases and potential regenerative therapeutics.

In a study by Elger *et al*[45] (2003) partial nephrectomy was performed to characterize kidney regeneration in *Leucoraja erinacea*, a species of skate[46]. Interestingly, upon resection a neonephrogenic zone was identified that resembles the mammalian embryonic metanephric kidney. This zone encompassed stem cell-like mesenchymal cells that were observed to aggregate around the CD tips. These cells proceeded to epithelialize and form cysts of varying morphologies, which appeared similar to mammalian metanephric structures such as RVs, comma-shaped bodies, and S-shaped bodies. The cysts progressively differentiated into distinct nephron segments, and vascularization of the glomerulus occurred. Neonephrogenesis not only occurred within the injured kidney, but also occurred within the uninjured contralateral kidney. Because neonephrogenesis in fish proceeds through similar stages as mammals, this suggests that genes regulating these events are conserved[38]. This study presents a possible model where renal stem cells persist in the adult kidney of skates and lower vertebrates[45].

In 2011, Diep *et al*[41] performed the first extensive molecular analysis of adult nephron progenitors in the adult zebrafish kidney and assessed their self-renewal capacity through transplantation studies as well. First, the researchers isolated whole-kidney marrow (WKM) cells from transgenic animals that express fluorescent reporters in the distal nephron. Upon transplant of these cells into immunocompromised, gentamicin treated recipients, many donor-derived nephrons were observed. The donor-derived nephrons were capable of blood filtration, indicating integration of the new structures in the recipient's tissue. These results support that renal progenitors are present in the adult zebrafish kidney and are able to engraft and give rise to new nephrons after transplantation. When a mix of $EGFP^+$ and $mCherry^+$ WKM cells was transplanted into conditioned recipients, mosaic nephrons resulted. This indicates that multiple progenitors can contribute to an individual nephron, consistent with similar data from mouse studies[16]. In addition, serial transplantation of WKM revealed that nephron progenitors are a self-renewing population possessing substantial proliferative

potential. It was determined that *lhx1a*$^+$ cellular aggregates are comprised of renal progenitors, and when these aggregates are ablated, nephrogenesis is terminated. Transplantation of a single *lxh1a*$^+$ cellular aggregate was sufficient to form multiple nephrons. This study illustrates *lhx1a*$^+$ progenitors in adult zebrafish act comparably to the Six2$^+$ CM cells during mammalian development. Although zebrafish *lhx1a*$^+$ progenitors and mouse Six2$^+$ progenitors possess distinct global gene expression profiles, several factors associated with renal development and stem cell potential were found to be conserved between the two cell populations. Using zebrafish as a model to elucidate molecular pathways regulating renal progenitors may be translatable in the establishment of novel stem cell therapies to treat human kidney diseases.

DISCUSSION

Chronic kidney disease (CKD) continues to be a problem that plagues our society, as it affects millions of individuals worldwide[47]. CKD can progress to end stage renal disease, which is ultimately an irreversible condition. The only treatment options for patients with end stage renal disease are organ transplant or dialysis[48]. This poses a serious problem, as the availability of donor organs is low and dialysis is not a permanent cure. In addition to CKD, a variety of developmental disorders affecting the renal and urinary tract exist[49]. Although these congenital conditions are rare, they involve severe kidney malformations that give rise to many health complications. Achieving a greater understanding of the dynamic biological mechanisms governing kidney development will unravel the mysteries of disease pathogenesis and lead to the discovery of innovative regenerative therapies.

The identification and characterization of adult renal progenitors paves the way for potential stem-cell therapies. Stem cell populations, like renal progenitors, are ideal targets for gene therapy, cell transplantation, and tissue engineering[50]. For example, it has been shown in various studies that the transplant of renal progenitors into injured rodents drives tissue repair and improves kidney functionality[20-22,27,29].

In addition to mice, zebrafish provide a unique model system to study kidney development and regeneration[51-53]. Zebrafish are incredible animals, as they are experts of kidney regeneration due to their extraordinary ability to undergo neonephrogenesis throughout their adult life, which can be induced further with well-established injury models[54-59]. Although vertebrates possess kidneys of varying organization and complexity, the genetic pathways that regulate organogenesis are highly conserved[60]. The diverse cell types that comprise the nephron are conserved across species, contributing to a growing appreciation of zebrafish as a relevant model system to study kidney development and regeneration. Furthermore, zebrafish may help identify novel genes regulating renal progenitors, neonephrogenesis, and regeneration. Future studies could determine factors essential for activating renal progenitors in adult zebrafish, which could potentially be translated to humans in order to induce these cells to facilitate tissue repair in disease contexts. The discovery of molecular mechanisms directing renal progenitor cell-fate decisions during development and regeneration holds great promise in advancing the fields of tissue engineering and stem-cell therapy.

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MINIREVIEWS

Mesenchymal stem cell therapy in retinal and optic nerve diseases: An update of clinical trials

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Abstract

Retinal and optic nerve diseases are degenerative ocular pathologies which lead to irreversible visual loss. Since the advanced therapies availability, cell-based therapies offer a new all-encompassing approach. Advances in the knowledge of neuroprotection, immunomodulation and regenerative properties of mesenchymal stem cells (MSCs) have been obtained by several preclinical studies of various neurodegenerative diseases. It has provided the opportunity to perform the translation of this knowledge to prospective treatment approaches for clinical practice. Since 2008, several first steps projecting new treatment approaches, have been taken regarding the use of cell therapy in patients with neurodegenerative pathologies of optic nerve and retina. Most of the clinical trials using MSCs are in Ⅰ/Ⅱ phase, recruiting patients or ongoing, and they have as main objective the safety assessment of MSCs using various routes of administration. However, it is important to recognize that, there is still a long way to go to reach clinical trials phase Ⅲ-Ⅳ. Hence, it is necessary to continue preclinical and clinical studies to improve this new therapeutic tool. This paper reviews the latest progress of MSCs in human clinical trials for retinal and

optic nerve diseases.

Key words: Mesenchymal stem cells; Cell therapy; Optic nerve diseases; Clinical trials; Retinal diseases

Core tip: Advances in the knowledge of neuroprotection, immunomodulation and regenerative properties of mesenchymal stem cells (MSCs) are contributed by several preclinical studies of various neurodegenerative diseases. It has provided opportunity to perform the translation of treatment approach to the clinical practice. Several clinical trials in patients with retinal and optic nerve diseases have been developed since 2008. Most of them using MSCs are in Ⅰ/Ⅱ phase. However, there is still a long way to go to reach clinical trials Phase Ⅲ-Ⅳ. Hence, it is necessary to continue with preclinical and clinical studies to improve this new therapeutic tool.

Labrador-Velandia S, Alonso-Alonso ML, Alvarez-Sanchez S, González-Zamora J, Carretero-Barrio I, Pastor JC, Fernandez-Bueno I, Srivastava GK. Mesenchymal stem cell therapy in retinal and optic nerve diseases: An update of clinical trials. *World J Stem Cells* 2016; 8(11): 376-383 Available from: URL: http://www.wjgnet.com/1948-0210/full/v8/i11/376.htm DOI: http://dx.doi.org/10.4252/wjsc.v8.i11.376

INTRODUCTION

Retinal dystrophies, diabetic retinopathy, age related macular degeneration and optic nerve diseases are chronic and degenerative ocular pathologies which lead to irreversible visual loss. Retinal degeneration is a leading cause of incurable low vision and blindness worldwide[1]. Most retinal and optic nerve diseases are caused by irreversible apoptosis of retinal neural cells or adjacent supporting tissue. Because there is no curative treatment for these degenerative diseases, current therapies mainly focus on the aetiology cause or at specific situations, such as late complications. However, most of them have low efficacy. Since the advanced therapies availability, cell-based therapies offer a new all-encompassing approach[2].

Mesenchymal stem cells (MSCs) are multipotent and self-renewing stem cells derived from bone marrow, adipose tissue, and other mesenchymal tissues, which can be induced to differentiate into bone marrow, cartilage, muscle, lipid, myocardial cells, glial cells and neurons[3,4]. MSCs have some features that make them useful in cell therapy research. These are easy to isolate and expand rapidly after a short period of dormancy[5]. They are free of ethical issues associated with the harvesting of embryonic stem cells[6]. Also, it is considered that MSCs are "immunoprivileged" because they do not express Major Histocompatibility Complex class Ⅱ (MHC-Ⅱ) on their surface, associated with transplant rejections[7], and this advantage allows its use as an autologous or allogenic form[8]. Furthermore, MSCs produce several growth factors with paracrine actions that are believed to modulate the microenvironment of diseased tissues, promote survival and activate endogenous repair mechanisms[9].

Due to this features MSCs have been used in several preclinical studies of retinal and optic nerve diseases, where they have demonstrated their properties of immunomodulation, neuroprotection and tissue repair[10-13]. These properties support the clinical use of MSCs as an opportunity for tissue repair and regeneration in several neurodegeneratives disorders. To remember, the stages of clinical trials for drugs in development can be divided into four phases. The main purpose of the first clinical stage, phase Ⅰ, is to observe the tolerance and pharmacokinetic characteristics of the drug in the human body and to provide evidence to establish the phase Ⅱ administration protocol. The purpose of phase Ⅱ clinical trials is to evaluate the efficacy and safety of the drug in patients with the target indication. In phase Ⅲ, the efficacy and safety of the drug in patients with the target indication is further validated, providing the basis of the evidence used for review during the drug registration and application process. The phase Ⅳ clinical trial, which takes place during the post marketing period, provides further evidence regarding the drug's efficacy and any emerging adverse reactions under conditions of real-life use in large numbers of patients[14].

In this review, we summarize the latest progress of MSCs in human clinical trials for retinal and optic nerve diseases.

TISSUE SOURCES OF MSCS

Bone marrow is the first isolation source of MSCs following by umbilical cord and adipose tissue[15]. Although bone marrow is the best source of obtaining MSCs, there are some aspects that reduced their use: Limited growth rate, differentiation capability depending on the donor age, and risk inherited to sample collection[15]. Regarding to umbilical cord source to obtain MSCs, it is required an optimal protocol such as, time of recollection and process less than 16 h, as well as, volume collection higher than 30 mL to get a success culture[16]. MSCs obtaining by adipose tissue source have a similar morphology and phenotype to the bone marrow source, but these cells have a higher capability of proliferation and adipose tissue samples are easier to collect from liposuction procedures[17].

CRYOPRESERVATION OF MSCS

Cryopreservation consists on the interruption of cellular metabolism regulated by processes of freezing and thawing, maintaining a good functional and structural cellular state. To preserve a biological sample as long as possible, without losing their properties, cells are immersed in liquid nitrogen at extremely low temperature

(-196 ℃), stopping the metabolic activity of the cells[18].

Cryopreservation has been performed primarily for the purpose of preserving the hematopoietic stem cell populations for transplantation. Currently, the use of this procedure has been extended, allowing the preservation of the biological potential, and to retain the biological age at time of cryopreservation. In autologous patients, MSCs are collected and cryopreserved for later clinical use. In allogeneic patients, cryopreservation permits banking of cells for human leukocyte antigen typing and matching, facilitating the logistical transport of cellular products to transplant centers, and allowing enough time for the screening of transmissible diseases in the donated cells before transplantation[19].

CLINICAL TRIALS USING MSCS

Today, there are ongoing clinical trials of advanced therapies' using MSCs in various retinal and optic nerve diseases. In these clinical trials the main route of administration is the intravitreal injection following by subretinal implant and then intravenous route. In all these studies it is used autologous stem cells from bone marrow or adipose tissue. On Table 1 it is shown all clinical trials finished and ongoing registered in clinicaltrials.gov and the International Clinical Trials Registry Platform, until today (Last search performed on 18 May 2016).

Clinical trials in retinal dystrophies: Retinitis pigmentosa and stargardt's disease

Retinitis pigmentosa (RP) includes some inherited diseases which are characterized by a classic pattern of difficulties in dark adaptation and night blindness in adolescence, loss of mid-peripheral visual field in young adulthood and central vision later in life due to the severe loss of rod and cone photoreceptors[20]. The RP is one of the leading hereditary degenerative retinal diseases, affecting 1 in 4000 individuals[20]. RP is characterized by the classic triad of decreased arteriolar diameter, pigment spicules deposits in the mid periphery of the retina and pallor of the papilla[20].

Stargardt's disease (SD) is the most common form of inherited juvenile macular degenerations. Its prevalence worldwide is estimated to be 1 in 10000 individuals[21]. Patients initially present with reduced central vision. The pathology is defined by the accumulation of lipofuscin in the apical zone of the RPE cells. The patients present decreased vision to legal blindness and secondary choroidal neovascularization, with bilateral gradual involvement of vision[21].

There are nine clinical trials that use MSCs to treat this kind retinal dystrophies (6 for RP, 2 for SD and RP and 1 for RP and other diseases) (Table 1). Although most clinical trials are in recruitment phase, there are two completed to treat retinitis pigmentosa, both were held at Hospital das Clinicas (Medical school Ribeirao Preto, Sao Paulo) - (NCT01068561 phase Ⅰ, NCT01560715 phase Ⅱ). The cells used were autologous bone marrow-derived MSCs, which were administered through intravitreal injection containing 10 × 10^6 cells/0.1 mL. The MSCs were obtained through aspiration of 10 mL bone marrow tissue from the posterior iliac crest and were separated by Ficoll-Hypaque gradient centrifugation. Regarding to the clinical trial NCT01068561 (phase Ⅰ), there is a case reported[22]. The case is about one recruited patient of this study, who had macular oedema associated with RP, which showed complete resolution of the oedema 7 d after injection, and the effect remained for one month of follow-up with optical coherence tomography. They concluded that the adult stem cells can restore the blood ocular barrier due their paracrine effects or by osmotic gradient allowing the absorption of macular oedema[22]. The trial NCT01560715 (phase Ⅱ) is completed and also have published results[23], they concluded that the therapy with intravitreal use MSC can improve the quality of life of patients with RP, although the improvement is lost with time. Patient's improvement has been evaluated with vision-related quality of life test (NEI VFG-25) before therapy and 3 and 12 mo later. There was a statistically significant improvement 3 mo after treatment, whereas by 12th month there was no significant difference from baseline[23].

At the hospital Virgen de la Arrixaca, Murcia (Spain), it is being carried out a phase Ⅰ clinical trial with autologous bone marrow stem cells in patients with RP. This clinical trial continues recruiting patients. Regarding to the other clinical trials for RP and Stargardt's disease (NCT01531348, NCT017336059, NCT01914913, NCT02280135, NCT027 09876 and NCT01518127), they are on phase Ⅰ or Ⅰ/Ⅱ, and they are recruiting patients (Table 1).

Clinical trials in diabetic retinopathy and age macular degeneration

Diabetic retinopathy (DR) is a prevalent microvascular complication of diabetes, and remains the leading cause of preventable blindness in working-aged people (20-74 years)[24]. About 30% all diabetics have signs of diabetic retinopathy, and 30% of these might have vision-threatening retinopathy, defined as severe retinopathy or macular edema[25]. The current standard treatment for management of these disorders relies mainly on laser therapy, which is inherently destructive, or antiangiogenic therapy, both associated with unavoidable ocular/systemic side-effects[25].

Age-related macular degeneration (AMD) is a progressive chronic disease of the central retina and a leading cause of vision loss worldwide, it accounts for 8% of all blindness worldwide and is the most common cause of blindness in developed countries[26], particularly in people older than 60 years. Its prevalence is likely to increase as a consequence of exponential population ageing. There have been significant advances in the management of exudative AMD with the introduction of anti-angiogenesis therapy, and patients now have effective treatment options that can prevent blindness and, in many cases, restore vision[27]. However antiangiogenic treatment doesn't stop the progression nor serves to treat dry AMD.

Table 1 Clinical trials for retinal and optic nerve diseases

Clinical trial	Condition	Cells	Route of administration	Dose	Estimated enrollment	Recruitment status	Study phase	Country	Start date
NCT01068561[1]	Retinosis pigmentaria	ABMSC	Intravitreal injection	10×10^6 cells/0.1 mL	5	Completed	I	Brazil	2010
NCT01531348	Retinosis pigmentaria	ABMMSC	Intravitreal injection	1×10^6 cells/0.1 mL	10	Enrolling by invitation	I	Tailandia	2012
NCT01560715[2]	Retinosis pigmentaria	ABMSC	Intravitreal injection	10×10^6 cells/0.1 mL	50	Recruiting	II	Brasil	2012
NCT01736059[3]	Retinosis pigmentaria, AMD, DR,VO	ABMSC	Intravitreal injection	3.4×10^6 cells/0.1 mL	15	Enrolling by invitation	I	EEUU	2012
NCT01914913	Retinosis pigmentaria	ABMSC	-	-	15	Recruiting	I / II	India	2014
NCT02280135	Retinosis pigmentaria	ABMSC	Intravitreal injection	30×10^6 cells/0.1 mL	10	Recruiting	I	Spain	2014
NCT02709876	Retinosis pigmentaria	ABMSC	Intravitreal injection	-	50	Recruiting	I / II	Arabia	2014
NCT01518127	Stargardt's disease and AMD	ABMSC	Intravitreal injection	10×10^6 cells/0.1 mL	10	Recruiting	I / II	Brazil	2011
NCT01736059[3]	Stargardt's disease,AMD, DR, VO, RP	ABMSC	Intravitreal injection	3.4×10^6 cells/0.1 mL	15	Recruiting	I	EEUU	2012
Carta al editor Act. Opht[4]	Diabetic retinopathy	ABMSC	Intravitreal injection	18×10^7 cells/0.5 mL	1	Completed	I	Germany	2008
NCT01518842	Diabetic retinopathy	ABMSC	Intravitreal injection	2×10^4 cells/0.1 mL	30	Unknown	I / II	Brasil	2011
IRCT201111291414N29	Diabetic retinopathy	ABMMSC	Intravenous	2×10^6 cells/kg	20	Ongoing	I / II	Iran	2011
NCT01736059[3]	Diabetic retinopathy, VO, HRD	ABMSC	Intravitreal injection	3.4×10^6 cells/0.1 mL	15	Recruiting	I	EEUU	2012
ChiCTR-ONC-16008055	Diabetic retinopathy	ASMSC	-	-	30	Recruiting	I / II	China	2013
NCT01518127	AMD, Stargardt's disease	ABMSC	Intravitreal injection	10×10^6 cells/0.1 mL	10	Recruiting	I / II	Brasil	2011
NCT01736059[3]	AMD, DR, VO, HRD	ABMSC	Intravitreal injection	3.4×10^6 cells/0.1 mL	15	Recruiting	I	EEUU	2012
NCT02016508	AMD	ABMSC	Intravitreal injection	-	1	Unknown	I / II	Egypt	2013
NCT02024269	AMD	AASC	Intravitreal injection	-	-	Withdrawn	I	EEUU	2013
NCT00787722	Neuromielitis óptica	AHSC	Intravenous	-	10	Recruiting	I	EEUU	2008
NCT01364246	Neuromielitis óptica	UC-MSC	Intravenous	-	20	Unknown	I / II	China	2010
NCT01339455	Neuromielitis óptica	AHSC	Intravenous	-	3	Ongoing	I / II	Canada	2011
NCT02249676	Neuromielitis óptica	ABMMSC	Intravenous	2×10^6 cells/kg	15	Recruiting	II	China	2014
NCT02638714	Optic nerve atrophy	AHSC	-	-	100	Ongoing	I / II	Jordania	2013
NCT01834079	Optic nerve atrophy	ABMSC	Intrathecal	10×10^7 cells/dose	24	Recruiting	I / II	India	2014
ChiCTR-TRC-14005093	Traumatic optic neuropathy	UC-MSC	Endonasal	-	70	Recruiting	I / II	China	2014
NCT02330978	Glaucoma	ABMMSC	Intravitreal injection	1×10^6 cells/0.1 mL	10	Recruiting	I	Brasil	2014
NCT02144103	Glaucoma	AASC	Subtenon injection	0.5 mL	16	Enrolling by invitation	I	Russia	2014
NCT01920867[5]	Retinal diseases, Macular degeneration, HRD, OND, glaucoma	ABMSC	Retrobulbar, subtenon, intravenous, intravitreal and intraocular injection	1.2×10^{12} cells/15 mL	300	Recruiting	I	Estados Unidos	2013

Last search performed in Clinicaltrials.gov and the International Clinical Trials Registry Platform, 18 May 2016. [1]Case reported[22]; [2]Case reported[23]; [3]Case reported[30]; [4]Case reported[28]; [5]Case reported[36,37]. ABMSC: Autologous bone-marrow stem cells; ABMMSC: Autologous bone-marrow mesenchymal stem cells; ASMSC: Autologous stromal mesenchymal stem cells; AASC: Autologous adipose stem cells; AHSC: Autologous hematopoietic stem cells; UC-MSC: Umbilical cord mesenchymal stem cells; AMD: Age-related macular degeneration; DR: Diabetic retinopathy; HRD: Hereditary retinal diseases; OND: Optic nerve diseases; RP: Retinitis pigmentosa; VO: Vein occlusions.

Thus, new approaches like stem cell therapy are needed.

The use of bone marrow derived stem cells (BMDSC) therapy for the DR has been evaluated[28,29] and there are five ongoing clinical trials (NCT01518842, IRCT 201111291414N29, NCT01736059, ChiCTR-ONC-16008055 and NCT01920867) (Table 1). In relation to this therapy for the AMD, it has been evaluated in four (4) ongoing clinical trials (NCT02016508, NCT01

920867, NCT01736059 y NCT01518127). One of them (NCT01736059) has published results in the AMD patients[30]. Bone marrow stem cells used in these clinical trials was harvested from the patient's own iliac crest (autologous use) with an average final volume of 50 mL (20-100 mL). Then, mononuclear cells were separated by Ficoll-gradient centrifugation. The dose of cells is between 2×10^4-1.8×10^8 suspended in 0.1 mL buffered saline solution. A trial using adipose derived stems cells (ADSC) has been withdrawn prior to enrollment (NCT02024269), however they don't explain the reasons.

Results of stem cell-treatment for the DR are limited to the report on two patients. A 43-year-old patient with very advanced atrophy of the retina and optic nerve caused by the DR and vision limited to defective light perception, after cell treatment patient have improvement but no signs of any side-effects, such as inflammation or infection[28]. The other reports a patient with macular oedema associated with macular ischemia, and describe the decrease of macular oedema and the improvement of retinal function after intravitreal injection of BMDSC[29].

Moreover, the only clinical results of MSCs therapy for the AMD[30] describes two patients who start from a visual acuity (VA) of 20/200. After intravitreal injection, they had an improvement with its new VA of 20/80 and 20/160. The patient with VA 20/80 kept it during first six months and the other patient with VA 20/160 worsened to its initial state of 20/200. A slight growth of extrafoveal geographic atrophy in both eyes of both patients was detected by fluorescein angiography. The results of electroretinography showed a slight worsening of the macular function of both eyes that could be attributed to the disease progression. In analysis by OCT hyperdense deposits were evident within the retinal layers after a month of therapy that correspond in size with $CD34^+$ cells, however, more studies are needed to prove whether it corresponds to intraretinal incorporation of $CD34^+$ cells. The results suggest that this cell therapy in patients with the AMD, especially in advanced stages, would not stop the progression[30].

Clinical trials of MSCs for optic neuropathies

Optic neuropathies are characterized by damage to the optic nerve and they can be due to various causes, such as glaucoma, autoimmune diseases, inflammation, infections, traumas, ischemia or compression. Glaucoma is the most common cause of optic nerve-related visual loss in adults, followed by nonarteritic anterior ischaemic optic neuropathy (NAION)[31]. The treatment for glaucoma is based on drugs and surgery that reduce intraocular pressure, whereas there is no treatment for NAION, nor to reverse the process nor for its recurrence[32]. Traumatic optic neuropathy is a cause of severe visual loss and it has no reliable treatment[33]. Neuromyelitis optica, also known as Devic's disease, is an autoimmune, demylienating disorder which causes optic neuritis. Its prevalence is about 1-3/100000[34]. Nowadays neuromyelitis optica treatment is based in corticosteroids and plasma exchange for the acute attacks and immunosuppressant drugs for the maintenance therapy[35].

Currently, there are two clinical trials at phase Ⅰ using MSCs to treat glaucoma (NCT02330978 and NCT02144103), both of them are recruiting patients at the moment. One of them is being held at Medical School Ribeirao Preto, University of São Paulo, Brazil (NCT02330978), and the other one in Burnasyan Federal Medical Biophysical Center, Russia (NCT02144103). The Brazilian one uses an intravitreal injection of 10^6 autologous bone marrow derived mesenchymal stem cells (BMMSCs) to assess the safety of the procedure and how it improves visual field and visual acuity. The Russian one uses a sub Tenon administration of autologous adipose-derived regenerative cells that have been extracted from the patient's front abdominal wall. There are still no published results of these studies.

In the SCOTS clinical trial (NCT01920867), held at the Johns Hopkins Hospital, United States, there is one case reported of autoimmune optic neuropathy[36]. They made a vitrectomy and intra-optic injection of autologous bone marrow stem cells (BMSCs) in one patient's eye and retrobulbar, sub Tenon and intravitreal injection in the other eye, improving the visual acuity, macular thickness and fast retinal nerve fiber layer thickness. In this clinical trial there is also a case reported of idiopathic bilateral optic neuritis[37]. The patient received a retrobulbar injection, sub Tenon injection and intravitreal injection of autologous BMSCs for the right eye (OD), and vitrectomy and direct intra-optic nerve injection of autologous BMSCs for the left eye (OS), followed by intravenous infusion. After this procedure, there was an improvement in visual acuity in both eyes and remained stable at the 12 mo post-operative[37].

For neuromyelitis optica there is one active clinical trial at Foothills Medical Centre, University of Calgary, Canada (NCT01339455), two recruiting patients at Northwestern University, United States (NCT00787722), one ongoing clinical trial in Tianjin Medical University General Hospital, China (NCT02249676), and one with unknown status at Nanjing University Medical College Affiliated Drum Tower Hospital, China (NCT01364246). Most of them, active and recruiting clinical trials, use immunosuppressive treatment followed by an autologous hematopoietic stem cells transplantation. While the Nanjing University uses human umbilical cord mesenchymal stem cells transplantation. In this clinical trial (NCT01364246), 5 patients were followed for 18 mo including evaluation of Expanded Disability Status Scale (EDSS) levels, clinical course, magnetic resonance imaging (MRI) characteristics and adverse events. and they reported an improvement in the symptoms and signs of neuromyelitis optica in four out of five patients treated[38]. There is another clinical trial for secondary progressive multiple sclerosis with evidence of optic nerve involvement (NCT00395200), in which patients were treated with autologous bone marrow stem cells transplantation and that resulted in an increase in visual acuity, visual evoked response latency, and optic nerve

area[39]. Some individual cases with neuromyelitis optica treated with allogeneic hematopoietic stem cells have been reported[40].

Traumatic optic neuropathy is being studied in a clinical trial in China, by the Cell Biotherapy Center, Daping Hospital, Third Military Medical University (ChiCTR-TRC-14005093). Currently, they are recruiting patients and will use human umbilical cord derived mesenchymal stem cells transplantation. There are still no results.

There are also clinical trials for optic neuropathies, without considering what caused it. One of them is currently active (NCT02638714) and is held by Stem Cells of Arabia, Jordan. The patients will be treated with a transplantation of purified adult autologous bone marrow derived $CD34^+$, $CD133^+$, and $CD271^+$ stem cells due to their diverse potentialities to differentiate into specific functional cell types to regenerate damaged optic nerves, supporting tissues and vasculature. They will use clinical-grade purification system (CliniMACS) and Microbeads to purify the target cell populations. There is another clinical trial on optic atrophy, currently recruiting patients (NCT01834079) in Chaitanya Hospital in Pune, India. Patients will receive three intrathecal injections of 100 million autologous bone marrow derived mononuclear cells per dose at intervals of 7 d. There are no results posted yet of these studies.

DISCUSSION

Advances in the knowledge of neuroprotective, immunomodulative and regenerative properties of MSCs are continuously generated by several preclinical studies *in vitro* and *in vivo* in animal models of various neurodegenerative diseases, including optic nerve and retinal diseases. It has given the opportunity to perform the translation of treatment approaches to the clinical practice. Since 2008, several first steps, projecting new treatment approaches, have been taken regarding the use of cell therapy in patients with neurodegenerative pathologies of optic nerve and retina. It is about Phase Ⅰ or Ⅰ/Ⅱ clinical trials, which have as main objective the safety assessment of MSCs using various routes of administration, where the main route used is the intravitreal injection.

Nevertheless, of the 24 clinical trials registered on clinicaltrials.gov, there are only 2 clinical trials finished, 3 are ongoing, 15 are in recruiting patients phase, 3 are in unknown state and 1 clinical trial has been withdrawn without knowing the reasons for this decision. Most of the results published to date, are reduced to 6 cases reported in various retinal/optic nerve pathologies, their number of patients is very low, and these are exceptional cases, so, there is not enough evidence to get any valid and scientific conclusion.

Furthermore, most of these clinical trials use autologous cells, obtaining by bone marrow aspirates, so the final content to be administered is a concentrate of mononuclear cells, containing a very small percentage of MSCs (0.1%)[15], only four clinical trials use a specific concentration of MSCs without added another cell type. It is surprising that, although MSCs derived from adipose tissue are easier to obtain and in a higher concentration[17], there are only 2 clinical trials using this cell type, and one of them has been withdrawn without explanation. Regarding the use of allogenic MSCs, is limited to 2 clinical trials, which use MSCs derived from umbilical cord, however, it is not known whether their patients will receive immunosuppressive therapy.

Regarding to cell dose used in various clinical trials, there is a great variation from one to another. There is no consensus regarding the calculation of cell dose for the use of these cells through intravitreal injection. The clinical trials which use mononuclear cells aspirate, the doses are usually high (between 3×10^6 cells/0.1 mL and 30×10^6 cells/0.1 mL), whereas clinical trials using a concentrated purified of MSCs, doses are lower (1×10^6 cells/0.1 mL). However, the information collected by clinical trials.gov and the International Clinical Trials Registry Platform not specify the cell dose calculation or the cell production process.

CONCLUSION

It is important to know the development of cell therapy in relation to its use in the clinical practice. However, it is also important to recognize that, there is still a long way to go to reach clinical trials phase Ⅲ-Ⅳ. One of the factors necessary to move forward is to establish unified criteria for the dose to be used, another important factor is the use of only MSCs without another cells added, because MSCs are immunoprivileged cells, and do not produce rejection. It is also important to use more frequently allogeneic MSC associated with cryopreservation processes. It can be the key to a better bioavailability of these cells, getting greater advantages of MSCs derived from adipose tissue, which are easier in obtaining and production. Therefore, it is necessary to continue preclinical and clinical studies to improve this new therapeutic tool.

Limitations

Most of the clinical trials using MSCs are in Ⅰ/Ⅱ phase, recruiting patients or ongoing. The information available in clinicaltrials.gov about the procedure obtaining cells or the dose used in each clinical trial is not described in all cases. Hence, there are not enough published results to have scientific evidence about the use of these cells in retinal and optic nerve diseases.

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ORIGINAL ARTICLE

Basic Study

Gene expression and pathway analysis of *CTNNB1* in cancer and stem cells

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Author contributions: Tanabe S performed the research and wrote the paper; Kawabata T analyzed the 3D structures of proteins; Aoyagi K, Yokozaki H and Sasaki H contributed critical revision of the manuscript for important intellectual content.

Institutional review board statement: Diffuse-type gastric cancer (GC) tissues were originally provided by the National Cancer Center Hospital after obtaining written informed consent from each patient and approval by National Cancer Center Institutional Review Board (ID: No.17-030). The existing data already available to the public were analyzed in the article.

Informed consent statement: Written informed consent from each patient were obtained.

Conflict-of-interest statement: To the best of the authors' knowledge, no conflict of interest exists.

Data sharing statement: The microarray data for mesenchymal stem cells and diffuse-type gastric cancer are available to the public in NCBI's Gene Expression Omnibus (GEO) database and are accessible via GEO Series accession number GSE7888 and GSE42252, respectively.

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Abstract

AIM

To investigate β-catenin (CTNNB1) signaling in cancer and stem cells, the gene expression and pathway were analyzed using bioinformatics.

METHODS

The expression of the catenin β 1 (*CTNNB1*) gene, which codes for β-catenin, was analyzed in mesenchymal stem cells (MSCs) and gastric cancer (GC) cells. Beta-catenin signaling and the mutation of related proteins were also analyzed using the cBioPortal for Cancer Genomics and HOMology modeling of Complex Structure (HOMCOS) databases.

RESULTS

The expression of the *CTNNB1* gene was up-regulated in GC cells compared to MSCs. The expression of EPH receptor A8 (EPHA8), synovial sarcoma translocation chromosome 18 (SS18), interactor of little elongation

complex ELL subunit 1 (ICE1), patched 1 (PTCH1), mutS homolog 3 (MSH3) and caspase recruitment domain family member 11 (CARD11) were also shown to be altered in GC cells in the cBioPortal for Cancer Genomics analysis. 3D complex structures were reported for E-cadherin 1 (CDH1), lymphoid enhancer binding factor 1 (LEF1), transcription factor 7 like 2 (TCF7L2) and adenomatous polyposis coli protein (APC) with β-catenin.

CONCLUSION

The results indicate that the epithelial-mesenchymal transition (EMT)-related gene *CTNNB1* plays an important role in the regulation of stem cell pluripotency and cancer signaling.

Key words: β-catenin; CTNNB1; Epithelial-mesenchymal transition; Mesenchymal stem cell; Stem cell

Core tip: β-catenin signaling consists of several pathway cascades, such as those that are involved in pluripotent stem cell generation and cancer. Several genes, including *EPHA8*, *SS18*, *ICE1*, *PTCH1*, *MSH3* and *CARD11*, are mutated along with *CTNNB1*. The expression of the *CTNNB1*, *CDH1*, *MYC*, *LEF1* and *TCF7L2* genes, which are related to the *CTNNB1* network, is up-regulated in diffuse-type GC cells compared to MSCs. 3D complex structures for β-catenin (CTNB1_HUMAN) with LEF_MOUSE and TF7L2_HUMAN were found using the HOMCOS database. The EMT-related gene *CTNNB1* plays an important role in pluripotent stem cell signaling and cancer signaling.

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INTRODUCTION

Changes in the phenotypes of cancer and stem cells are related to changes in gene expression and protein signaling. This study aims to reveal the β-catenin (*CTNNB1*) regulation in diffuse-type gastric cancer (GC) cells and mesenchymal stem cells (MSCs). Wnt/β-catenin signaling is necessary for epithelial-mesenchymal transitions (EMT)[1]. Stem cell division is strongly correlated with cancer risk, and this highlights the importance of molecular signaling in stem cells and cancer cells[2]. Epigenetics and stem cell functions are regulated by several exogenous stimuli, including cell-cell and cell-matrix interactions[3]. To ensure the safety of therapeutic stem cell applications in terms of stem cell modification, an understanding of the regulation of the stem cells and their niche is necessary[4]. In the case of bone metastasis, the tissue-specific stromal response for prostate cancer can be identified by a molecular signature for which a novel mechanism has been revealed in hematopoietic and prostate epithelial stem cell niches[5].

Cancer stem cell (CSC) maintenance requires hypoxia-inducible factor (HIF)-α transcription factors and the inhibitor of DNA binding 2 (ID2)[6]. The down-regulated expression of ID2 is associated with a poor prognosis in hepatocellular carcinoma[7].

Because the compendium of gene expression, chromosomal copy number and sequencing data from human cancer cell lines, which is called the Cancer Cell Line Encyclopedia (CCLE), has revealed that genomic data are capable of predicting anti-cancer drug sensitivity, molecular and network analyses should be carried out[8]. It has been reported that cadherin 1 (*CDH1*) is up-regulated in diffuse-type GC cells compared to MSCs[9]. However, *CDH2* was down-regulated in diffuse-type GC cells compared to MSCs; this provides a useful indicator - the ratio of *CDH2* to *CDH1* expression - to distinguish the mesenchymal and epithelial phenotypes of the cells[9]. It has been reported that catenin β 1 (*CTNNB1*) is mutated in hepatocellular carcinoma[10,11]. To further elucidate the EMT phenotype and the molecules that are involved in β-catenin signaling in cancer, the *CTNNB1* network and the β-catenin binding partners have been investigated in this report using bioinformatics tools such as microarray analysis and databases.

MATERIALS AND METHODS

Gene expression analysis of MSCs and diffuse-type GC cells

Gene expression in MSCs (n = 12) and diffuse-type GC cells (n = 5) was analyzed using Human Genome U133 Plus 2.0 microarrays, as previously described[9,12]. In brief, total RNA was purified from the cells, biotinylated and hybridized to microarrays. The signal intensity of each gene transcript was analyzed and compared between MSCs and diffuse-type GC cells. The microarray data for MSCs and diffuse-type GC cells are available to the public in NCBI's Gene Expression Omnibus (GEO) database and are accessible *via* GEO Series accession number GSE7888 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE7888) and GSE42252 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE42252), respectively[9,12].

Diffuse-type GC tissues

Diffuse-type GC tissues were originally provided by the National Cancer Center Hospital after obtaining written informed consent from each patient and approval by National Cancer Center Institutional Review Board. All cancer specimens were reviewed and classified histopathologically according to the Japanese Classification of Gastric Cancer. Tissue specimens were immediately frozen with liquid nitrogen after surgical extraction, and stored at -80 ℃ until microarray analysis[9,13]. The existing data

Table 1 3D complex structures of β-catenin (CTNNB1) and interacting proteins

pdb_id	β-catenin (CTNNB1)			Proteins that interact with β-catenin					
	ChainID	Length	UniProtID	Molecule	ChainID	Length	UniProtID	Contact protein name	Regulation of gene expression in GC cells compared to MSCs
1th1	B	513	CTNB1_HUMAN	APC	D	54	APC_HUMAN	Adenomatous polyposis coli protein	Not changed/-
1qz7	A	524	CTNB1_HUMAN	AXIN1	B	17	AXN_XENLA	Axin-1	-
3sl9	B	165	CTNB1_HUMAN	BCL9	D	23	BCL9_HUMAN	B-cell CLL/ lymphoma 9 protein	-
1i7w	C	509	CTNB1_MOUSE	CDH1	D	60	CADH1_MOUSE	Cadherin-1	Up-regulated
1m1e	A	512	CTNB1_MOUSE	CTNNBIP1	B	65	CNBP1_HUMAN	Beta-catenin-interacting protein 1	-
3oux	A	503	CTNB1_MOUSE	LEF1	B	47	LEF1_MOUSE	Lymphoid enhancer-binding factor 1	Up-regulated
3tx7	A	504	CTNB1_HUMAN	NR5A2	B	218	NR5A2_HUMAN	Nuclear receptor subfamily 5 group A member 2	-
1g3j	A	439	CTNB1_HUMAN	TCF7L1	B	34	T7L1A_XENLA	Transcription factor 7-like 1-A	-
1jdh	A	508	CTNB1_HUMAN	TCF7L2	B	38	TF7L2_HUMAN	Transcription factor 7-like 2	Up-regulated
1dow	B	32	CTNB1_MOUSE	CTNNA1	A	205	CTNA1_MOUSE	Catenin alpha-1	Not changed/-
4ons	D	56	CTNB1_MOUSE	CTNNA2	C	230	CTNA2_MOUSE	Catenin alpha-2	-

already available to the public were analyzed in the article.

Analysis of cancer genomics using cBioPortal

The cancer genomics data analysis was performed relative to *CTNNB1* using the cBioPortal for Cancer Genomics (http://www.cbioportal.org)[14,15]. The term "CTNNB1" was searched in the cBioPortal for Cancer Genomics database, and a cross-cancer alteration summary was obtained for *CTNNB1*. A study on stomach adenocarcinoma was further analyzed for enrichments[16]. Genes with mutations that were enriched in samples that contained altered *CTNNB1* were selected in the cBioPortal for cancer genomics for further study.

3D complex structures

3D complex structures were searched in the HOMology modeling of COmplex Structure (HOMCOS) database (http://homcos.pdbj.org) using the search engine that was provided by the VaProS server (http://pford.info/vapros)[17]. The UniProtID "CTNB_HUMAN" was input as the query for the "searching contact molecule" field of the HOMCOS. Only close homologues (sequence identity > 95%) were selected. The complex structures that were found were superimposed using the MATRAS program[18].

Statistical analysis

The data were expressed as the mean ± SE. For the statistics, Student's *t* test was used. $P < 0.01$ was considered as statistically significant.

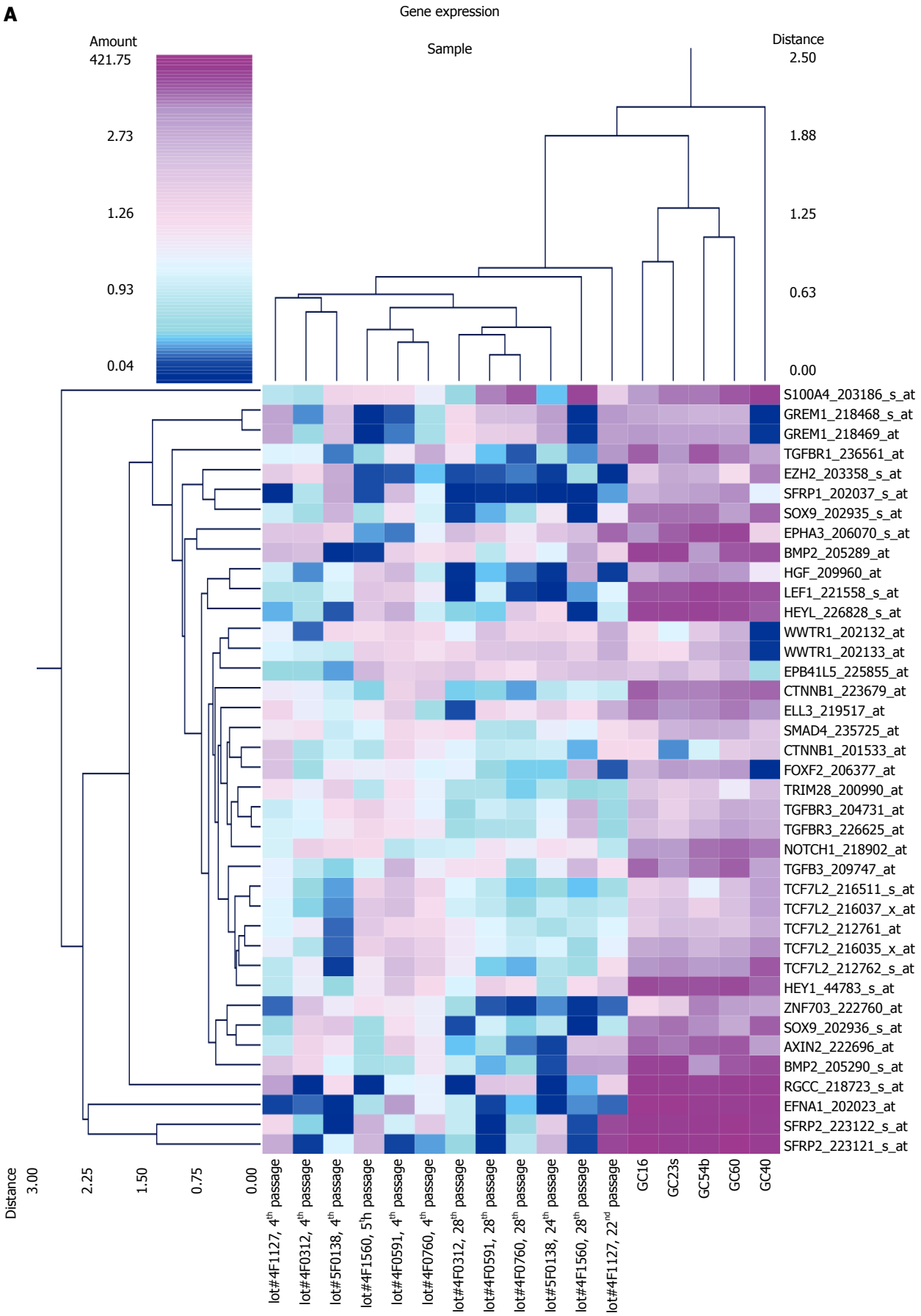
RESULTS

Expression of EMT-related genes in MSCs and diffuse-type GC cells

The expression of EMT-related genes in MSCs and diffuse-type GC cells is shown in Figure 1. The genes for which probe sets included the "EMT" term in the Gene Ontology (GO) Biological Process field were selected as EMT-related genes. The average signal intensity for early-stage MSCs, late-stage MSCs, or GC cells was greater than 500. Panel A shows the results of a cluster analysis of 39 probe sets that were up-regulated in diffuse-type GC cells compared to early-stage MSCs (n = 6 in early-stage MSCs, n = 6 in late-stage MSCs, n = 5 in GC). Panel B shows the results of a cluster analysis of 46 probe sets that were down-regulated in diffuse-type GC cells compared to early-stage MSCs (n = 6 in early-stage MSCs, n = 6 in late-stage MSCs, n = 5 in GC). To evaluate *CTNNB1* expression in cancer and stem cells, the expression of the *CTNNB1* gene was compared in MSCs and diffuse-type GC cells, and the results indicate that *CTNNB1* is up-regulated in GC cells (Figure 2). One of the probe sets was up-regulated more than 8-fold over its expression level in MSCs, whereas the other probe sets showed no increases in expression in GC cells compared to MSCs.

3D complex structures of β-catenin

To verify and explore protein-protein interactions with β-catenin, 3D complex structures of β-catenin were found using the HOMCOS database (http://homcos.pdbj.org)[17] and are summarized in Table 1. Figure 3 shows the superimposed 3D structure of the complex. Most of the proteins bind to the inner concave surface of the armadillo repeat region of β-catenin by using their 40-60 residue length extended peptides [adenomatous polyposis coli protein (APC), E-cadherin 1 (CDH1), catenin beta interacting protein 1 (CTNNBIP1), lymphoid enhancer binding factor 1 (LEF1), transcription factor 7 like 1 (TCF7L1) and transcription factor 7 like 2 (TCF7L2)].


A
Gene expression
Sample
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0.93
0.04
Distance
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1.25
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0.00
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EFNA1_202023_at
SFRP2_223122_s_at
SFRP2_223121_s_at
Distance
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2.25
1.50
0.75
0.00
lot#4F1127, 4th passage
lot#4F0312, 4th passage
lot#5F0138, 4th passage
lot#4F1560, 5h passage
lot#4F0591, 4th passage
lot#4F0760, 4th passage
lot#4F0312, 28th passage
lot#4F0591, 28th passage
lot#4F0760, 28th passage
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GC16
GC23s
GC54b
GC60
GC40

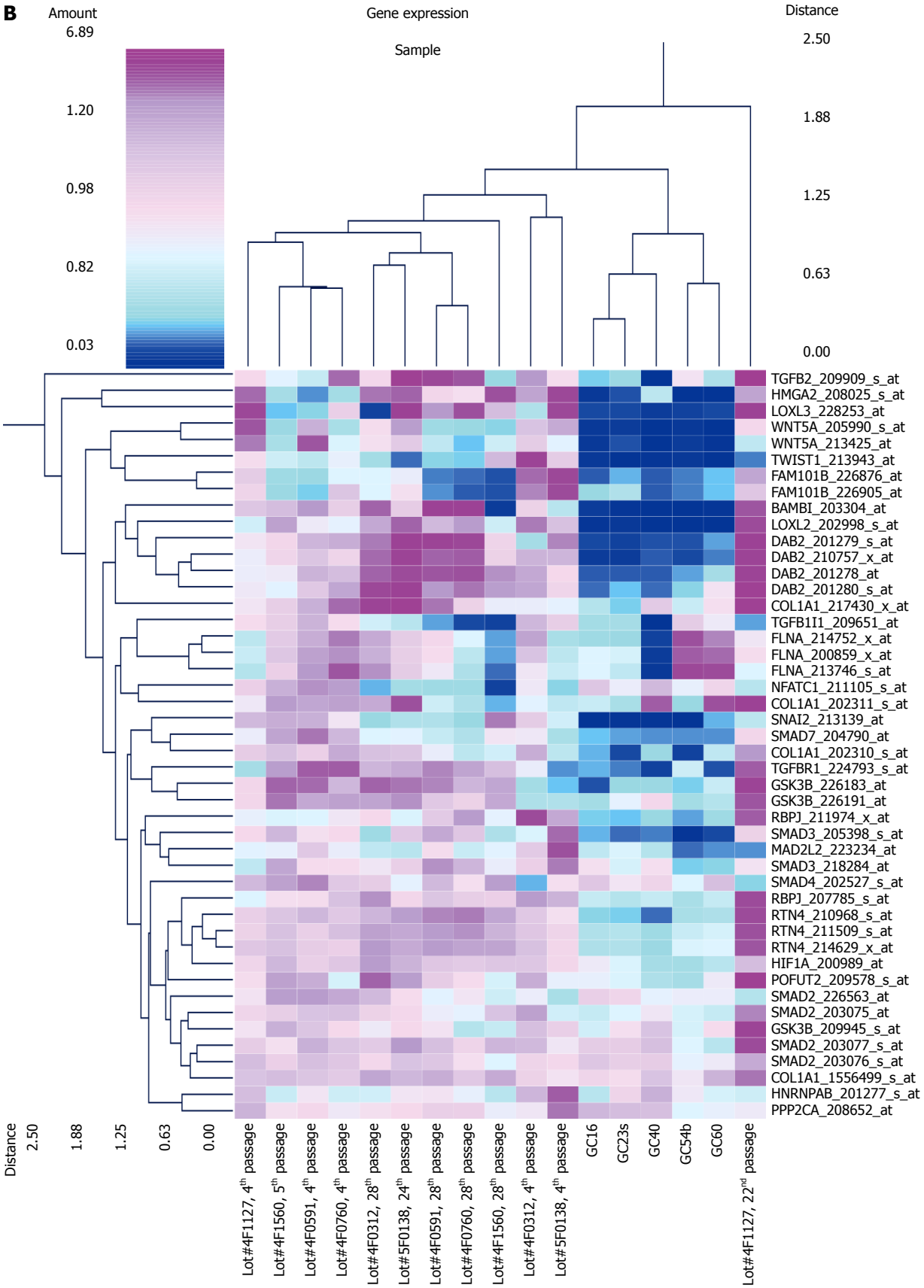

Figure 1 Expression of epithelial-mesenchymal transition-related genes in mesenchymal stem cells and diffuse-type gastric cancer cells. Cluster analysis of gene expression in mesenchymal stem cells (MSCs) and diffuse-type gastric cancer (GC) cells. A: The result of the cluster analysis of 39 probe sets that were up-regulated in diffuse-type GC cells compared to early-stage MSCs (n = 6 in early-stage MSCs, n = 6 in late-stage MSCs, n = 5 in GC); B: The result of the cluster analysis of 46 probe sets that were down-regulated in diffuse-type GC cells compared to early-stage MSCs (n = 6 in early-stage MSCs, n = 6 in late-stage MSCs, n = 5 in GC). The probe sets with epithelial to mesenchymal transition in the Gene Ontology Biological Process were selected (the average signal intensity in early-stage MSCs, late-stage MSCs, or GC cells is greater than 500).

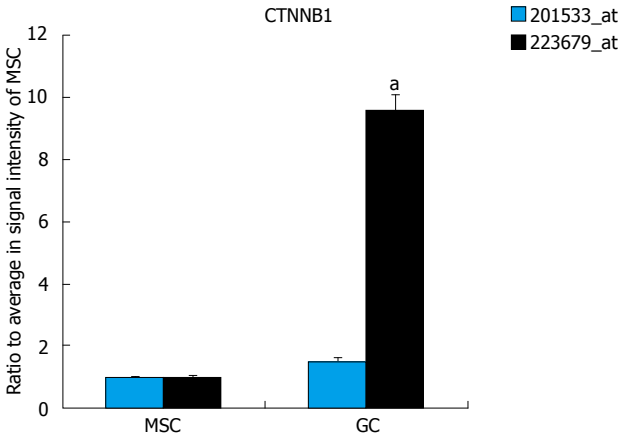

Figure 2 *CTNNB1* expression in mesenchymal stem cells and diffuse-type gastric cancer cells. *CTNNB1* gene expression was up-regulated in GC cells compared to MSCs. The signal intensity of probe set ID 223679_at was up-regulated more than 8-fold in GC cells compared to MSCs, whereas the signal intensity in probe set ID 201533_at was unchanged (n = 12 in MSC, n = 5 in GC, $^{a}P < 0.01$ in t test). GC: Gastric cancer; MSCs: Mesenchymal stem cells.

CDH1 and B-cell CLL/lymphoma 9 (BCL9) bind to the N-terminal region of the repeat that has the alpha-helical peptides. The nuclear receptor subfamily 5 group A member 2 (NR5A2) ligand binding domain binds to the middle of the armadillo repeat region. Of these binding factors, the transcription of the *CDH1*, *LEF1* and *TCF7L2* genes was up-regulated in GC cells (Table 1). It has been reported that a small molecule antagonist of the β-catenin/T-cell transcription factor 4 [TCF4; official name is transcription factor 7 like 2 (TCF7L2)] interaction inhibits self-renewal of CSCs and suppresses tumorigenesis[19]. The 3D complex structures of β-catenin and TCF7L2 are available[20,21]. The complex structure of NR5A2 has also been reported[22]. NR5A2 (or liver receptor homolog-1; LRH1) is a member of the nuclear hormone receptor family of transcription factors that play essential roles in development, metabolism, and cancer and are implicated in Wnt/β-catenin signaling[22]. NR5A2 is essential for the early development and maintenance of pluripotent mouse embryonic stem (ES) cells[22,23]. Network models for CTNNB1, the Wnt signaling pathway, Hippo signaling pathway and adherens junction signaling in cancer are shown in Figure 4. CTNNB1 binds to CDH1 near the cellular membrane or to TCF to transcribe anti-apoptotic or pro-proliferation genes, such as SRY-box 2 (*SOX2*) or v-myc avian myelocytomatosis viral oncogene homolog (*MYC*) (Figure 4). Wnt stimulation prevents glycogen synthase kinase 3 beta (GSK3β) from phosphorylating CTNNB1 and leads to CTNNB1 translocation into the nucleus to induce transcription. The 3D complex structure (PDB code: 1m1e) clearly shows how CDH1 binds to CTNNB1 in the mouse model.

CTNNB1 pathway (Kyoto Encyclopedia of Genes and Genomes)

CTNNB1 is listed in 21 pathways in Kyoto Encyclopedia of Genes and Genomes (KEGG), including the Rap1 signaling pathway, Wnt signaling pathway, Hippo signaling pathway, focal adhesion regulation, adherens junction regulation, tight junction regulation, signaling pathways that regulate the pluripotency of stem cells, leukocyte transendothelial migration, melanogenesis, the thyroid hormone signaling pathway; bacterial invasion of epithelial cells, pathogenic *Escherichia coli* infection, HTLV-I infection, and various cancer pathways. The following conditions use the aforementioned pathways and are also thus implicated: Proteoglycans in cancer, colorectal cancer, endometrial cancer, prostate cancer, thyroid cancer, basal cell carcinoma, and arrhythmogenic right ventricular cardiomyopathy (ARVC) (http://www.genome.jp/dbget-bin/www_bget?hsa:1499). The inhibition of GSK3β kinase activates β-catenin, which stimulates endoderm induction *via* the degradation of Tcf7l1 and forkhead box A2 (FoxA2) expression[24]. Wnt signaling induces intracellular β-catenin signaling *via* GSK3β kinase inhibition and dephosphorylation of β-catenin[25-28]. The inhibition of β-catenin decreases proliferation and induces apoptosis in the mantle cell lymphoma cell line[29]. Noncanonical Wnt signaling is activated in circulating tumor cells from the prostate that are anti-androgen-resistant[30].

Mutations in *CTNNB1* and related genes (cBioPortal: Stomach adenocarcinoma)

The Cancer Genome Atlas Research Network project has indicated that there is a characteristic molecular signature for ras homolog family member A (*RHOA*) mutations in diffuse type stomach adenocarcinoma[16]. Two-hundred and ninety-five primary gastric adenocarcinomas have been investigated, and mutations in *RHOA* have been enriched in genomically stable subtype, diffuse-type GC cells[16]. The analysis with cBioPortal showed that *CTNNB1* was altered in 24 (8%) of 287 cases/patients in stomach adenocarcinoma: 4 amplifications, 2 deep deletions, 12 missense mutations, 5 truncating mutations and 1 inframe mutation. Several gene mutations occurred concurrently with *CTNNB1* alterations in stomach adenocarcinoma (Table 2). The development of mutations in EPH receptor A8 (*EPHA8*), synovial sarcoma translocation chromosome 18 (*SS18*), interactor of little elongator complex ELL subunit 1 (*ICE1*), patched 1 (*PTCH1*), mutS homolog 3 (*MSH3*) and caspase recruitment domain family member 11 (*CARD11*) occurred alongside the *CTNNB1* alterations (Table 2). Of the mutated genes, *PTCH1* expression was up-regulated in GC cells compared to MSCs (Table 2). The GO of the mutated genes is shown in Table 3. EPHA8 possesses kinase activity, SS18 is involved in cell morphogenesis, ICE1 may play a role in positive regulation of intracellular protein transport, PTCH1 is involved in morphogenesis and cell growth, MSH3 is involved in mismatch repair, and CARD11 regulates B cell proliferation, apoptosis and NF-κB signaling, according to GO biological process (Table 3). GO biological process terms in Table 3 are based on Affymetrix annotation (http://www.affymetrix.com/estore/) and gene information in NCBI (http://www.ncbi.nlm.nih.gov/).

β-catenin signaling model

Several β-catenin-binding proteins, such as LEF1 or

Table 2 Genes mutated along with the *CTNNB1* alteration

Gene symbol	Gene title	Cytoband	Mutation percentage		Log ratio	P-value	Ratio of GC cells to MSCs
			In altered group	In unaltered group			
EPHA8	EPH receptor A8	1p36.12	29.17%	2.28%	3.68	1.45E-05	Signal intensity is low
SS18	Synovial sarcoma translocation Chromosome 18	18q11.2	16.67%	0.00%	> 10	3.84E-05	0.6 1.4
ICE1	Interactor of little elongator complex ELL subunit 1	5p15.32	33.33%	4.56%	2.87	4.74E-05	1.5
PTCH1	Patched 1	9q22.3	29.17%	3.42%	3.09	8.16E-05	16.6
MSH3	MutS homolog 3	5q14.1	20.83%	1.14%	4.19	1.28E-04	Signal intensity is low
CARD11	Caspase recruitment domain family, member 11	7p22	29.17%	4.18%	2.8	2.03E-04	Signal intensity is low

Table 3 Gene ontology of mutated genes along with *CTNNB1* alteration

Gene symbol	Gene ontology biological process
EPHA8	Protein phosphorylation // substrate-dependent cell migration // cell adhesion // transmembrane receptor protein tyrosine kinase signaling pathway // multicellular organismal development // nervous system development // axon guidance // phosphorylation // neuron remodeling // peptidyl-tyrosine phosphorylation // regulation of cell adhesion // neuron projection development // regulation of cell adhesion mediated by integrin // positive regulation of MAPK cascade // positive regulation of phosphatidylinositol 3-kinase activity // protein autophosphorylation // ephrin receptor signaling pathway
SS18	Microtubule cytoskeleton organization // cell morphogenesis // transcription, DNA-templated // regulation of transcription, DNA-templated // cytoskeleton organization // response to drug // positive regulation of transcription from RNA polymerase II promoter // ephrin receptor signaling pathway
ICE1	Positive regulation of intracellular protein transport // positive regulation of protein complex assembly // positive regulation of transcription from RNA polymerase III promoter // snRNA transcription from RNA polymerase II promoter // snRNA transcription from RNA polymerase III promoter
PTCH1	Negative regulation of transcription from RNA polymerase II promoter // branching involved in ureteric bud morphogenesis // neural tube formation // neural tube closure // heart morphogenesis // signal transduction // smoothened signaling pathway // smoothened signaling pathway // regulation of mitotic cell cycle // pattern specification process // brain development // negative regulation of cell proliferation // epidermis development // regulation of smoothened signaling pathway // response to mechanical stimulus // organ morphogenesis // dorsal/ventral pattern formation // response to chlorate // positive regulation of cholesterol efflux // response to organic cyclic compound // protein processing // spinal cord motor neuron differentiation // neural tube patterning // dorsal/ventral neural tube patterning // neural plate axis specification // embryonic limb morphogenesis // mammary gland development // response to estradiol // response to retinoic acid // regulation of protein localization // limb morphogenesis // hindlimb morphogenesis // regulation of growth // negative regulation of multicellular organism growth // regulation of cell proliferation // response to drug // glucose homeostasis // negative regulation of sequence-specific DNA binding transcription factor activity // keratinocyte proliferation // negative regulation of osteoblast differentiation // negative regulation of smoothened signaling pathway // negative regulation of smoothened signaling pathway // negative regulation of epithelial cell proliferation // negative regulation of cell division // pharyngeal system development // mammary gland duct morphogenesis // mammary gland epithelial cell differentiation // smoothened signaling pathway involved in dorsal/ventral neural tube patterning // cell differentiation involved in kidney development // somite development // cellular response to cholesterol // cellular response to cholesterol // renal system development // cell proliferation involved in metanephros development // protein targeting to plasma membrane
MSH3	Meiotic mismatch repair // ATP catabolic process // DNA repair // mismatch repair // cellular response to DNA damage stimulus // reciprocal meiotic recombination // somatic recombination of immunoglobulin gene segments // maintenance of DNA repeat elements // negative regulation of DNA recombination // positive regulation of helicase activity
CARD11	Positive regulation of cytokine production // signal transduction // positive regulation of B cell proliferation // T cell costimulation // Fc-epsilon receptor signaling pathway // positive regulation of T cell proliferation // regulation of apoptotic process // positive regulation of I-kappaB kinase/NF-kappaB signaling // thymic T cell selection // positive regulation of interleukin-2 biosynthetic process // innate immune response // regulation of B cell differentiation // regulation of T cell differentiation // nucleotide phosphorylation // regulation of immune response // T cell receptor signaling pathway // positive regulation of T cell activation // positive regulation of NF-kappaB transcription factor activity

TCF7L2, share high mobility group (HMG)-box domains, which suggests that β-catenin signaling switches mechanisms with the binding of different transcription factors. 3D complex structures show that CDH1, LEF1 and TCF7L2 bind to β-catenin. The role of β-catenin signaling in the pluripotentcy pathway should be investigated to reveal its mechanism in cancer and stem cells. The Wnt pathway is located upstream, and TCF, downstream of CTNNB1 in the cascade[31]. The merged network model of the β-catenin signaling network and CDH1, together with molecules in the 3D complex structures and genes mutated along with the *CTNNB1* alteration is shown in Figure 5A. The merged network model of the *CTNNB1*, *Wnt*, and *TCF* signaling networks and *CDH1*, together with molecules in the 3D complex structures and genes mutated along with the *CTNNB1* alteration is shown in Figure 5B. Of

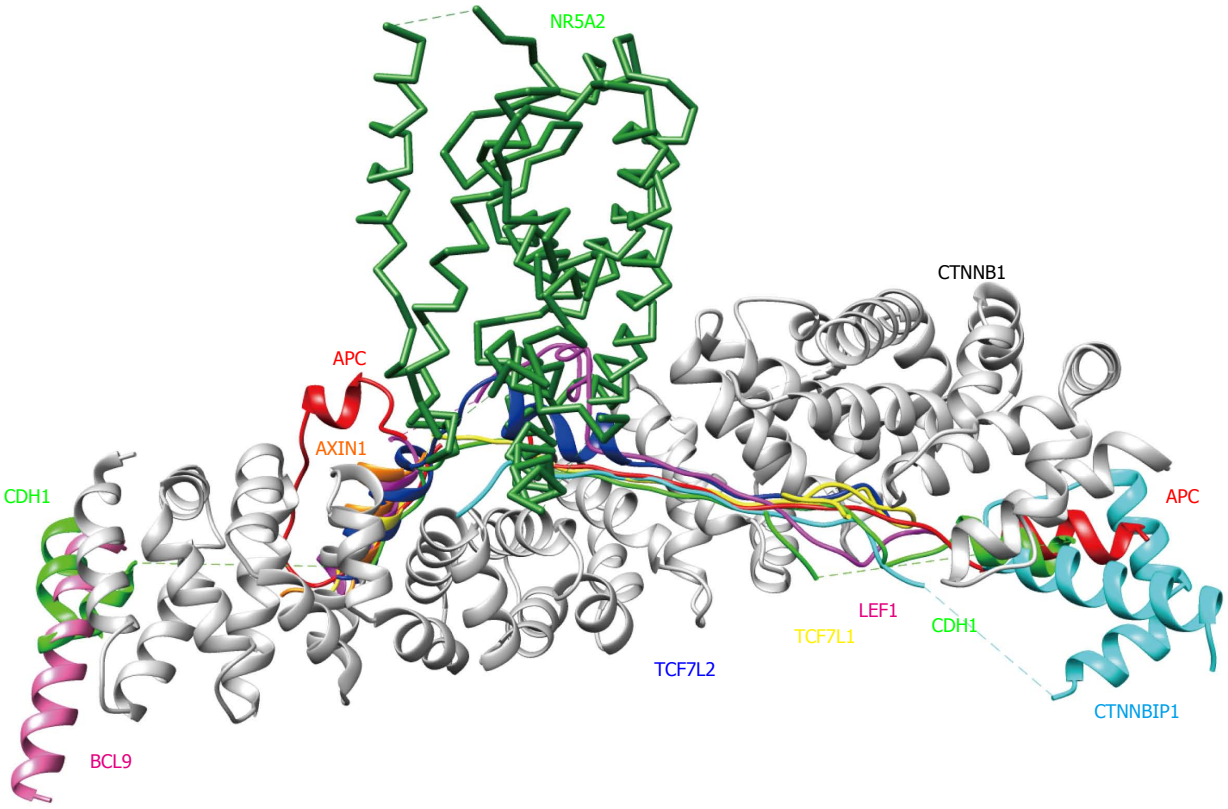

Figure 3 3D structures of β-catenin (CTNNB1) binding proteins. Each CTNNB1 complex structure was superimposed onto the CTNNB1 structure in a complex with APC (PDB code: 1th1), using the program MATRAS (From Ref. [18]). Colors and PDB codes are summarized as follows: White: CTNNB1 (CTNB1_HUMAN, 1th1); red: APC (APC_HUMAN, 1th1); orange: AXIN1 (AXN_XELNA, 1qz7); hot pink: BCL9 (BCL9_HUMAN, 3sl9); green: CDH1 (CADH1_MOUSE. 1i7w); cyan: CTNNBIP1 (CNBP1_HUMAN, 1m1e); magenta: LEF1 (LEF1_MOUSE, 3oux); forest green: NR5A2 (NR5A2_HUMAN, 3tx7); yellow: TCF7L1 (T7L1A_XENLA, 1g3j); blue: TCF7L2 (TF7L2_HUMAN, 1jdh).

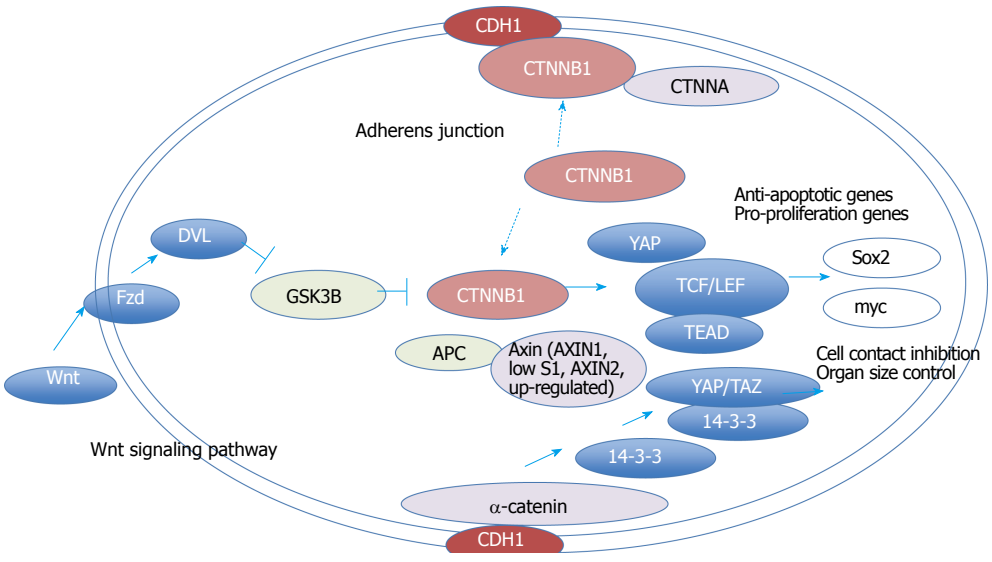

Figure 4 Network model for CTNNB1. The molecular network model for CTNNB1 signaling is shown. The extracted networks for pathways in cancer, Hippo signaling pathway and the Wnt signaling pathway (KEGG) were merged and are shown in a molecular network model. Wnt signaling and adherens junction molecules cross-talk *via* CTNNB1. Activated CTNNB1 induces the transcription of anti-apoptosis genes and pro-proliferation genes.

the common genes, EPHA8, SS18 and PTCH1 interact with phosphatidylinositol-4,5-bisphosphate-3-kinase catalytic subunit gamma (PIK3CG), SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 4 (SMARCA4), and GLI family zinc finger 1 (GLI1), respectively, whereas CARD11, ICE1, MSH3 have no known interactions with molecules in the CTNNB1 network. The networks for stomach

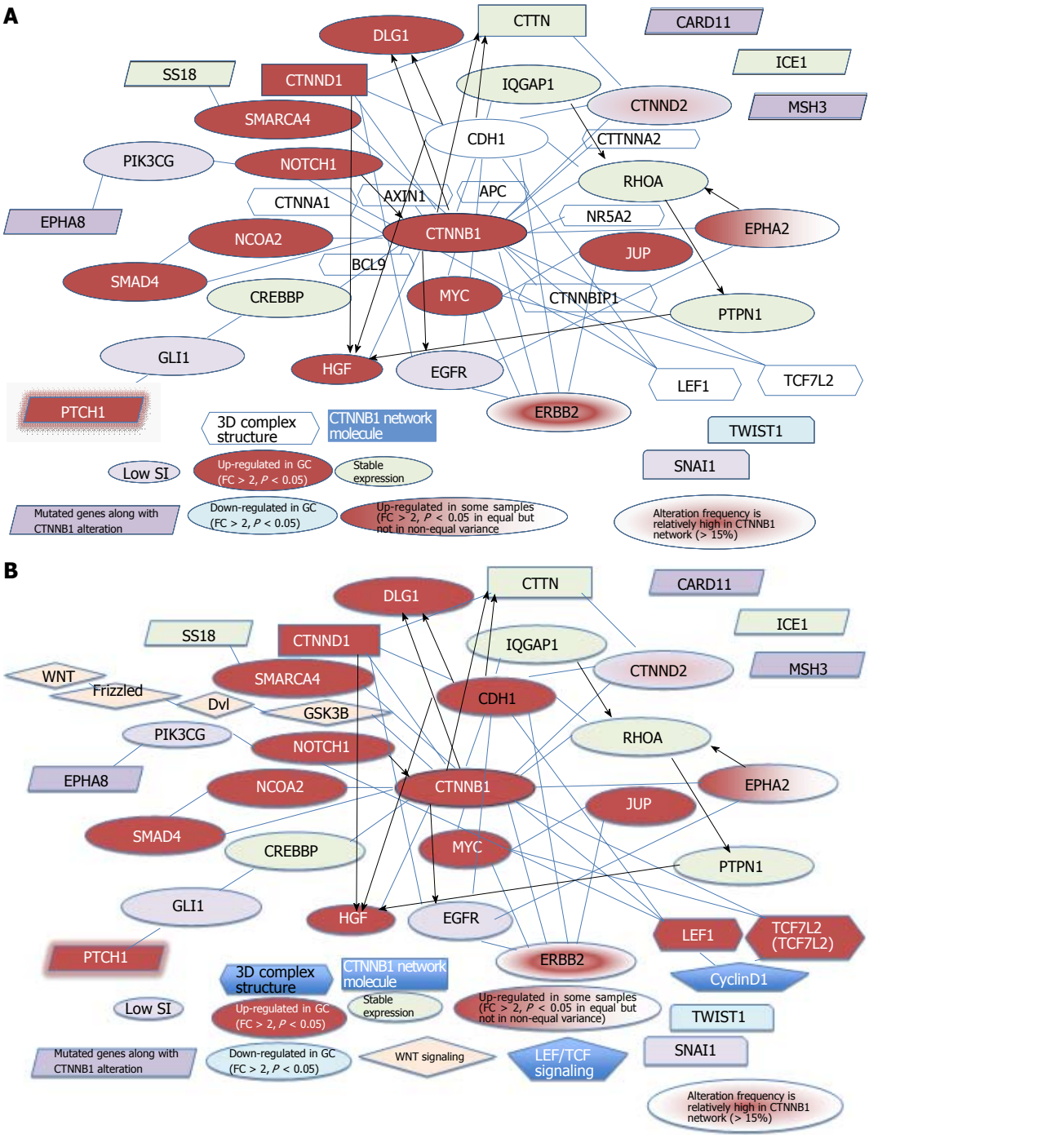

Figure 5 Network model for *CTNNB1* and related genes. A: Network model for *CTNNB1* and genes mutated along with *CTNNB1*. The networks of extracted *CTNNB1* with other mutated genes plus that of extracted *CTNNB1* alone are shown (cBioPortal-oriented, Stomach Adenocarcinoma)[16]; B: Network model for *CTNNB1* and 6 mutated genes. Wnt and LEF/TCF signaling are merged in *CTNNB1* signaling (cBioPortal-oriented, Stomach Adenocarcinoma)[16].

adenocarcinoma that were generated using cBioPortal for Cancer Genomics for CTNNB1 alone and for CTNNB1 with the 6 genes that are mutated along with the *CTNNB1* alteration have been partially merged in Figure 5. Catenin delta 2 (*CTNND2*) and erb-b2 receptor tyrosine kinase 2 (*ERBB2*) showed a relatively high frequency of mutation (> 15% in 287 tumor samples) in the analysis using cBioPortal for Cancer Genomics of the *CTNNB1* network in stomach adenocarcinoma (TCGA, Nature 2014)[16]. The genes that were up-regulated in GC cells compared to MSCs are shown in red, whereas the down-regulated genes are shown in light blue (Fold change > 2, $P < 0.05$, $n = 12$ in MSCs, $n = 5$ in GC; the average signal intensity of MSCs or GC cells is greater than 500). The expression of the *CTNNB1*, *CDH1*, notch1 (*NOTCH1*), hepatocyte growth factor (*HGF*), *PTCH1*, discs large homolog 1, scribble cell polarity complex component (*DLG1*), *LEF1*, *CTNND1*, *SMARCA4*, nuclear receptor coactivator 2 (*NCOA2*), SMAD family member 4 (*SMAD4*), *MYC*, junction plakoglobin (*JUP*), *TCF7L2* and *ERBB2* genes

was up-regulated in GC cells compared to MSCs, whereas the expression of twist family bHLH transcription factor 1 (*TWIST1*) was down-regulated in GC cells compared to MSCs. The expression of *EPHA2* was up-regulated in some GC samples. The expression of the IQ motif-containing GTPase activating protein 1 (*IQGAP1*), *SS18*, *ICE1*, cortactin (*CTTN*), *RHOA*, CREB binding protein (*CREBBP*) and protein tyrosine phosphatase, non-receptor (*PTPN1*) genes was not altered in MSCs and GC cells. The expression of the *EPHA8*, *PIK3CG*, *CARD11*, *MSH3*, *GLI1*, epidermal growth factor receptor (*EGFR*), snail family zinc finger 1 (*SNAI1*) and *CTNND2* genes was not examined due to a low signal intensity. The alteration frequencies of *CTNND2* and *ERBB2* are relatively high in the CTNNB1 network (> 15%), according to the cBioPortal for Cancer Genomics. Interestingly, *IQGAP2* was up-regulated in GC cells compared to MSCs.

DISCUSSION

In summary, the *CTNNB1* gene expression was up-regulated in diffuse-type GC compared to MSC. The various molecules are regulated with *CTNNB1*, which suggests the *CTNNB1* signaling network in cancer and stem cells. EMT-related genes have been reported to be induced by transforming growth factor (TGF)-β or epidermal growth factor (EGF), and genes in the Wnt signaling pathway are mutated in non-small cell lung cancer[32-34]. The expression of β-catenin was up-regulated in the TGF-β1-induced EMT model and was inhibited by cucurbitacin B treatment[32]. Solid tumors induce hypoxia, leading to HIF-1α protein regulation of molecules that are involved in angiogenesis, erythropoiesis, metabolism, cell survival and cell proliferation[35]. *SNAI2* and *TWIST1* were down-regulated in GC cells compared to MSCs, whereas *SNAI1* expression was not detected because of low signal intensity[9,36,37]. Because SNAI and TWIST are associated with EMT, the regulation of their expression is important for understanding EMT mechanisms. Although 3D complex structures of SNAI2 and TWIST1 with β-catenin are not available, some indirect β-catenin signaling cascade may be involved in the SNAI2 and TWIST1 pathway[38,39]. TGFβ is also an important factor in EMT[40]. TGFβ regulates osteoblast differentiation, whereas calycosin-7-O-β-D-glucopyranoside-induced osteoblast differentiation is regulated *via* the bone morphogenetic protein (BMP) and Wnt/β-catenin-signaling pathway[41]. The TGFβ-induced nuclear translocation of β-catenin has been reported to be one of the key factors that activates the EMT program[42-45]. Wnt/β-catenin is regulated in stem cells, and Wnt target genes are controlled by the TCF/β-catenin complex[46].

In gastrointestinal cancer, somatic mutations that provoke an immune response have been found in tumor-infiltrating lymphocytes, which may be very specific to the individual and are targets for cancer immunotherapy[47]. KRAS-mutation-specific T cells, as well as personalized mutation-specific T cells, have been identified, and these may be useful in the future for individual cancer immunotherapeutics[47]. It has been reported that *Helicobacter pylori* up-regulates Nanog and Oct4 expression *via* Wnt/β-catenin signaling[48]. Wnt/β-catenin signaling and the phosphorylation of β-catenin may be involved in stemness in gastric cancer[48].

In conclusion, CTNNB1 plays an important role in the regulation of stem cell pluripotency and cancer signaling. For future direction, precise analyses of Wnt signaling, Notch signaling, and Ephrin signaling are needed to reveal the entire picture of β-catenin signaling in cancer and stem cells. RHO mutations, and regulator of G-protein signaling, with network analysis tools, such as Cytoscape, must be investigated for a greater understanding of this process.

ACKNOWLEDGMENTS

The authors would like to thank Professor Kei Yura for introducing the VaProS database. This study used VaProS, a data-cloud developed by the Information Core of the Platform Project for Supporting Drug Discovery and Life Science Research (Platform for Drug Discovery, Informatics, and Structural Life Science) from the Japan Agency for Medical Research and Development (AMED).

COMMENTS

Background

β-catenin signaling is essential in pluripotent stem cells and cancer. It is also involved in the epithelial-mesenchymal transitions (EMT). CTNNB1 is activated by Wnt, and the binding of CTNNB1 to transcription factors leads to pluripotent gene regulation.

Research frontiers

The regulation of pluripotency and proliferation is important for elucidating the mechanism of cell phenotype transitions. The EMT mechanism should be investigated to better understand cancer resistance to therapeutics.

Innovations and breakthroughs

The 3D complex structures of β-catenin and related molecules were studied using molecular networks, which is an innovation in the field. The mutated genes that were altered along with *CTNNB1* in stomach adenocarcinoma samples were also investigated.

Applications

These results may affect the study of the pluripotency mechanism and potential therapeutic predictions of gastric cancer. The genes in the molecular network that are related to *CTNNB1* may be the targets of predictive medicine for cancer and disease using pluripotent cells.

Terminology

EMT is a cellular phenotype of a transition from an epithelial to a mesenchymal cell type. EMT is regulated in cancer metastasis and malignancy, and it is related to the acquisition of resistance in cancer cells to therapeutics. It is important to understand the EMT mechanism to understand the mechanisms of cancer resistance.

Peer-review

In general, the manuscript is interesting not only for scientific reasons, but also due to its potential clinical relevance, since it provides some light about the

relationships between stem and cancer cells.

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