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Research Article

ADMINISTRATION OF BONE MARROW DERIVED MESENCHYMAL STEM CELLS MODULATE TLR EXPRESSION DURING LIVER REGENERATION

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Abstract: Liver cell transplantation is a powerful alternative to orthotopic cell transplantation in the treatment of liver failures. Recently, considerable effort is being channeled to understand the nature and kinetics of directing stem cells to effectively accumulate at the regenerating liver site. Mesenchymal stem cells are one of the promising cell sources modulating liver regeneration process. The present was designed to study how mesenchymal stem cells might modulate liver immune behaviors by changing Toll-like receptor (TLR) expression and increase regenerative potential during liver regeneration in rats.

Normal and partially hepatectomized rats were treated with mesenchymal stem cells isolated and expanded from rat bone marrows. Accumulation of mesenchymal stem cells was confirmed by Real Time-Polymerase Chain Reaction (RT-PCR), Fluorescence-Activated Cell Sorting (FACS), and Immunofluorescence Staining (IFS). Student's t-test analysis was used to evaluate the significance of differences between sham and partially hepatectomized rat groups.

Our results showed that mesenchymal stem cells expressed several TLRs, and their accumulation during regeneration was depended on the timing of injury. Mesenchymal stem cells isolated from bone marrow of normal rats were observed at the injured liver 3 days after the injection. There were no labeled mesenchymal stem cells in the liver sections of the uninjured animals. Mesenchymal stem cell administration significantly altered the expression of TLR2, 3 and 9 while retaining their migration potential to regenerating liver.

Our findings implicated that mesenchymal stem cell administration during liver regeneration modulate the immune response through changing the expression of the TLRs in the remaining liver parts into which the cells are recruited or infused. This alteration may contribute to the regeneration process following partial hepatectomy.

Key words: Mesenchymal stem cell, TLR, homing, liver regeneration, rat.

Özet: Karaciğer hücresi nakli, karaciğer yetmezliğinde ortotopik hücre nakline güçlü bir alternatiftir. Son yıllarda, kök hücrelerin doğalarının, kinetiklerinin ve yenilenen karaciğer bölgesinde etkili bir şekilde toplanmalarının sağlanmasının anlaşılması için hatırı sayılır gayretler sarf edilmektedir. Mesenkimal kök hücreler karaciğer yenilenme sürecini modüle eden ümit verici hücre kaynaklarından bir tanesidir. Bu çalışma, mezenkimal kök hücrelerin sıçanlarda toll benzeri reseptör (TLR) ifadesini değiştirmek suretiyle karaciğer immün yanıtını nasıl etkileyebildiklerini ve karaciğer yenilenmesi esnasında yenilenme potansiyelini arttırabildiklerini belirlemek için gerçekleştirilmiştir.

Normal ve karaciğerleri kısmen çıkarılmış sıçanlar, sıçan kemik iliğinden elde edilip çoğaltılan mesenkimal kök hücreler ile muamele edilmişlerdir. Mesenkimal kök hücrelerin toplanması Eş Zamanlı Polimeraz Zincir Reaksiyonu (RT-PCR), Floresan Aktivite Hücre Ayırma (FACS), ve Immunfloresan Boyama (IFS) ile doğrulanmıştır. Sham ve karaciğeri kısmen alınmış sıçan grupları arasındaki farklılığın istatistiki analizinde Student's t-testi kullanılmıştır.

Elde edilen sonuçlar mezenkimal kök hücrelerinde çeşitli TLR'lerin ifade edildiklerini ve bu hücrelerin yenilenme esnasında toplanmalarının meydana gelen hasarın zamanlamasına bağlı olduğunu göstermiştir. Normal sıçanların kemik iliğinden izole edilen mezenkimal kök hücreler hasarlı karaciğerde enjeksiyon sonrası 3. günde görülmüşlerdir. Hasarsız hayvanların karaciğer kesitlerinde işaretli bir mezenkimal kök hücre görülmemiştir. Mezenkimal kök hücre uygulaması TLR2, 3 ve 9'un ifadesinin anlamlı bir şekilde değiştirirken yenilenen karaciğere göç etme yeteneklerini devam ettirmişlerdir.

Sonuçlar, karaciğer yenilenmesi esnasında mezenkimal kök hücre uygulamasının, hücrelerin uygulandığı hasarsız karaciğer parçalarında TLR'lerin ifadelerini değiştirme yoluyla immün yanıtı modüle ettiğini ortaya koymaktadır. TLR ifadesindeki bu değişim kısmı hepatoktemi sonrası yenilenme sürecine katkı sağlayabilir niteliktedir.

Introduction

Since its first development in the early 1960s (Starzl *et al*. 1963), orthotopic liver transplantation (OLT) has been considered as a gold standard in treatment of liver failure. However, other therapeutic strategies are also required due to the donor shortage. Despite its limiting factors such as cell viability, modest engraftment and limited tissue viability (Phillippe *et al*. 2008), liver cell transplantation (LCT) is one of the developing alternative solution with increasing success (Najimi & Sokal 2005, Stéphenne *et al.* 2006). Stem cells are very potent candidates for LCT due to their plasticity (Verfaillie *et al*. 2002). Besides hematopoietic stem cells (HSCs) and adult liver stem/progenitor cells, mesenchymal stem cells (MSCs) are also used in liver cell therapy because of their hepatic differentiation potential (Phillippe *et al*. 2008).

MSCs are multipotent cells capable of self-renewal and differentiating into multiple lineages such as osteocytes, adipocytes, chondrocytes, myoblasts, hepatocytes and cardiomyocytes (Friedenstein *et al.* 1970, da Silva Meirelles *et al.* 2008, Tokcaer-Keskin *et al.* 2009). In addition to their hepatic potential, MSCs have two other important properties that make them critical for LCT. The first one is their preferential migration into the injured site. Migration of MSCs and their engraftment after the injury have been shown in several tissues including myocardium (Orlic *et al.* 2001) spinal cord (Hofstetter *et al.* 2002), brain (Kopen *et al.* 1999), kidney (Bussolati *et al.* 2008) and liver (Zhao *et al.* 2008). Secondly, MSCs can evade immune recognition and were implicated to suppress immune responses (Rasmusson 2006). Through multiple pathways, *ex-vivo* expanded MSCs were reported to down regulate a broad range of innate and adaptive immune cells including T cells, B cells, NK cells and antigen presenting cells (Stagg 2006) demonstrating their versatile immunoregulatory properties (Bartholomew *et al.* 2002, Aggrawal & Pitteger 2005, Inoue *et al.* 2006). Although the mechanism is unclear yet, there seems to be several factors playing role in this immunoregulation. Pevsner-Fisher *et al.* (2007) showed that murine MSCs express several Toll-like receptors (TLRs) and in particular TLR2 was found to be important in differentiation potential of MSCs. It was also recently shown that human MSCs express several TLRs (Pervsner-Fischer *et al.* 2007, Tomchuck *et al.* 2008).

Cell surface or endosome-associated TLRs recognize a wide range of pathogen-associated molecular patterns (PAMPs) including carbohydrates, lipids, proteins and nucleic acids (Akira *et al.* 2006). Several immune cells such as macrophages, dendritic cells (DCs), B cells, specific types of T cells but also non-immune cells including fibroblasts and epithelial cells (Kumagai *et al.* 2008) signaling through TLRs were reported (Takeshita *et al.* 2004). In all cell types, expression of TLRs is modulated in response to pathogens, cytokines and environmental stress. While TLR1, 2, 4, 5 and 6 are expressed on the cell surface, TLR3, 7, 8 and 9 are found in intracellular compartments such as endosomes (Arancibia *et al.* 2007). Ligands of the latter ones, mainly nucleic acids, are required to be internalized to endosomes for recognition.

The collaborative role of TLRs on MSCs' homing during liver regeneration has not been evaluated so far. By using a well established liver regeneration model of partially hepatectomized (PH) rats, the present study was undertaken to investigate the interplay between MSCs and TLRs. Our findings implicated that MSC administration causes an increase in the expression of TLR2, 3 and 9 in the remaining liver and may facilitate MSC homing during liver regeneration.

Materials and Methods

Animals and the experimental design

Nine-weeks-old, 280–300 g, male Sprague–Dawley rats were obtained from the Experimental Animal Center of Bilkent University. The rats were housed in wire mesh cages under optimum laboratory conditions (temperature 22 ^{o}C±2 ^{o}C, humidity 50-55 %, light-dark period: 12h/12h) and daily fed with tap water and pellet foods, including % 21 pure proteins. All experimental protocols were approved by the Local Animal Ethics Committee of Bilkent University. Our protocol complied with Bilkent University's guidelines on the humane care and use of laboratory animals.

Animals were divided randomly in 3 groups as isolation group of MSCs, normal group (sham; SH) and partially hepatectomized (PH) group (Fig. 1). In the PH group, 70 % of the liver mass was resected (Higgins *et al.* 1931), and in the normal group, identical surgical procedures were carried out without resection after injecting 30 mg/kg ketamine (Ketalar, Pfizer, Istanbul, Turkey). Three animals per group were used in the experiments. Operations were performed between 08:00 and 12:00 hours to minimize diurnal effects. For mRNA expression experiments, animals from both groups were killed by cervical dislocation at 2 h, 4 h, 12 h, 18 h, and 24 h and after the operation. The remnant liver lobes were excised and immediately frozen in liquid nitrogen.

Isolation and culture labeling of MSCs and administration to PH rats

MSCs were also obtained from male, nine-week-old, 280–300 g Sprague–Dawley rat bone marrow (BM) according to the methods described elsewhere (Tokcaer-Keskin *et al.* 2009). $2x10^6$ of MSCs were treated with Chloromethylbenzamido-1, 1–dioctadecyl- 3,3,3',3'-tetramethylindocarbocyanine perchlorate (CM-DiI) (Molecular Probes, USA) at a concentration of 5 μg/mL in 1X PBS. Four days after CM-DiI labeling, cells were removed and $1x10^6$ cells/mL labeled MSCs were injected to the normal rats to obtain SH and partially hepatectomized PH groups (Fig. 1). Animals were sacrificed by cervical dislocation at 1, 3, and 5 days following partial hepatectomy. The remnant liver lobes were excised and immediately frozen in liquid nitrogen.

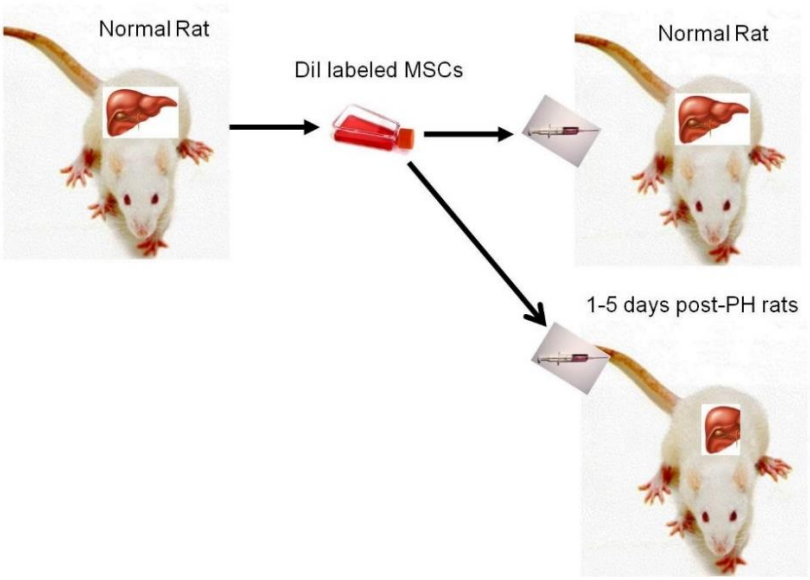

Fig. 1. MSCs were isolated and expanded from BM. CM-DiI labeled MSCs were injected into the normal and hepatectomized animals.

Total RNA Isolation from Rat MSCs and Liver Tissues

Total RNA from MSCs was isolated from the cell precipitate by using RNeasy Mini Kit (Qiagen, Hilden, Germany) and from liver tissues by using TriPure solution (Roche, Indiana, USA) according to the manufacturer's protocol. The cDNAs were synthesized with the DyNAmo cDNA synthesis kit (Finnzymes, Espoo, Finland) according to the manufacturer's protocol.

RT-PCR

RT-PCR conditions for CD11b, CD29, CD34, CD45, CD71, CD73, CD90, CD105, CD166 and GAPDH as the house keeping gene including corresponding primer sets are listed in Table 1. RT-PCR was done with DyNAmo™ HS SYBR® Green qPCR Kit (Finnzymes) according to the manufacturer's protocol.

Table 1. Primer sequences for RT-PCR, conditions and product sizes.

Gene Acronyms		Primer Sequences	Product Size (bp)	Cycle #
CD11b*[a]	Forward Reverse	GCTGGGAGATGTGAATGGAG TGATGCTGGCTACTGATGCT	113	30
CD29**[a]	Forward Reverse	ACTTCAGACTTCCGCATTGG GCTGCTGACCAACAAGTTCA	190	26
CD34**[b]	Forward Reverse	TGTCTGCTCCTTGAATCT CCTGTGGGACTCCAACT	281	30
CD45**[a]	Forward Reverse	ATGTTATTGGGAGGGTGCAA AAAATGTAACGCGCTTCAGG	175	26
CD71[$]	Forward Reverse	ATGGTTCGTACAGCAGCAGA CGAGCAGAATACAGCCATTG	182	35
CD73*[a]	Forward Reverse	GAACTTGGGAGGGAGGAGAG CATTGGCAGGAAGAGAGGAG	282	30
CD90**[b]	Forward Reverse	CCAGTCATCAGCATCACTCT AGCTTGTCTCTGATCACATT	374	30
CD105*[a]	Forward Reverse	CGGGAGGTGTTTCTGGTCT GTGTCTGGGTTCGTGGTTG	331	30
CD166*[a]	Forward Reverse	CTTTGTTCTGGGAGTGGCTG GGTGTTGCCGTATGTGTTTG	303	30
GAPDH*[a]	Forward Reverse	AGACAGCCGCATCTTCTTGT CTTGCCGTGGGTAGAGTCAT	207	30

** Initial Denaturation 95°C 10', Denaturation 94°C 40'', Extension 72°C 40'', Final Extension 72°C 5'.*
*** Initial Denaturation 95°C 5', Denaturation 94°C 30'', Extension 72°C 30'', Final Extension 72°C 10'.*
[a] Annealing 60°C 30'', [b] Annealing 55°C 30''.
[$] Initial Denaturation 95°C 5', Denaturation 94°C 45'', Annealing 66°C 60'', Extension 72°C 45'', Final Extension 72°C 10'.

Real-Time RT-PCR Studies

Rat TLR primer sequences were adopted from Hubert *et al.* (2006) and CYC was used as the house keeping gene. Efficiency of all primers were tested, standard curves were derived and E values were calculated (Table 2). The C*t* values of the *i)* normal liver and PH groups (post PH at 2, 4, 12, 18 and 24 hours) for *TLR* genes and *CYC* and *ii)* PH groups (normal liver vs. 1 and 3 days post-PH) and MSC-administered PH groups (normal liver vs. 1 and 3 days post-PH) for *TLR2, 3*, and *9* genes and *CYC* were calculated. By using normalized C*t* values, Δ*Ct* (ΔCt_{TLR} / ΔCt_{CYC}) for each *TLR* at indicated time was calculated. Fold changes in the expression of the tested TLR genes werecalculated by using the $2^{-(\Delta CtPH - \Delta CtNL)}$ formula. Real-time RT-PCR reactions followed by a melting curve analysis were carried out in iCycler™ (Bio-Rad, Hercules, CA, USA). Q-RT-PCR conditions for all investigated genes have an initial denaturation 95°C, 10 minutes followed by 40 cycles of denaturation for 15 seconds at 95°C, annealing for 60 seconds at 60°C, followed by extension of 60 seconds at 60°C. Final extension was set at 72°C for 5 minutes. Samples from PH and SH groups obtained at different time points were detected in duplicates, and readings from each sample and its internal control were used to calculate gene expression level.

Cell Surface Marker Staining and Analyses by FACS

Staining protocol was slightly modified from previously published protocol (Gursel *et al.* 2006). Rat specific antibodies against CD90-FITC (Santa Cruz Biotechnology, CA, USA), and CD45-PE/Cy5 (Abcam, Cambridge, UK) with isotype controls were used to characterize MSCs. The cells were incubated in dark for 30 minutes and were washed twice, resuspended in 500 µl PBS-BSA-Na azide and analyzed in FACSCalibur (BD, USA). FACS dot plots were assessed by Cell Quest Pro software.

Immunofluorescence Staining (IFS)

5 µm sections of frozen liver tissues were fixed in 4 % paraformaldehyde for 30 minutes and were immersed in 3 % H_2O_2 (in methanol v/v) for 30 minutes. After washing with phosphate buffered saline pH: 7.2-7.4 (PBS), the specimen was blocked with 2 % bovine serum albumin (BSA) for 1 hour at room temperature (RT) in a humid chamber. Sections were then incubated with anti-FLT3 (Santa Cruz Biotechnology) and anti-CD90 (Chemicon, Temecula, Canada) antibodies at RT for one hour at a dilution of 1/50 and 1/500 in 1 % BSA, respectively. The tissues were incubated with FITC-labeled anti-mouse IgG (Sigma, St. Louis, MO, USA) and FITC-labeled anti-rabbit IgG (Sigma) for CD90 and FLT3, respectively. After incubating in dark for 1 hour at RT, the specimens were mounted using UltraCruz™ (Santa Cruz) medium with DAPI and examined under fluorescence microscope.

Statistical Analysis

Statistical significance between PH and SH groups was determined using the Student's *t*–test analysis. *P*-value <0.05 was considered to be statistically significant.

Table 2. The sequences of different rat TLR primers (adopted from Hubert *et al.* 2006) and efficiency values of the primer sets (E value).

Gene Acronyms		Primer Sequences	Product Size (bp)	E value
TLR1	Forward Reverse	CAGCAGCCTCAAGCATGTCT CAGCCCTAAGACAACAATACAATAGAAGA	82	1.94
TLR2	Forward Reverse	CTCCTGTGAACTCCTGTCCTT AGCTGTCTGGCCAGTCAAC	74	1.94
TLR3	Forward Reverse	GCACTGTGAGATACAACGTAGCT GAAGGTCATCAGGTATGTGTGTCA	66	1.98
TLR4	Forward Reverse	TGCTACAGTTCATCTGGGTTTCTG CTGTGAGGTCGTTGAGGTTAGAAG	78	1.88
TLR5	Forward Reverse	GGGCAGCAGAAAGACGGTAT CAGGCACCAGCCATCCTTAA	61	1.86
TLR6	Forward Reverse	AGAACCTTACTCATGTCCCAAAAGAC AGATCAGATATGGAGTTTTGAGACAGACT	79	1.99
TLR7	Forward Reverse	GTTTTACGTCTACACAGTAACTCTCTTCA TTCCTGGAGGTTGCTCATGTTTT	75	2.00
TLR8	Forward Reverse	GGCTTCGGCAGAGGATCT GCCAAAACAAGTTTTCCGCTTTG	75	2.00
TLR9	Forward Reverse	CCGAAGACCTAGCCAACCT TGATCACAGCGACGGCAATT	70	2.00
TLR10	Forward Reverse	CTCCAACATGGCTTTAAGGAAGGT TGGAATTGATAGAGGAGGTTGTAGGA	90	1.90
CYC	Forward Reverse	GGGAGGGTGAAAGAAGGCAT GAGAGCAGAGATTACAGGGT	211	1.90

Results

Characterization of MSC by PCR and by FACS

We first isolated MSCs from rat bone marrow. Consistent with previous observations by us and others (Tokcaer-Keskin *et al.* 2010, Mangi *et al.* 2003, Pittenger *et al.* 1999), the cells were positive for MCS markers such as CD90, CD71, CD73, CD29, CD105 and CD166 and negative for hematopoietic cell lineage markers such as CD11b, CD34 and CD45 at the transcript level (Fig. 2A). We further confirmed that these MSCs were positive for CD90 (84.5±7.2 % of total population, MFI: 334.5±150.3) and negative for CD45 (96.6±1.2 % of total population, MFI: 10.4±0.5) by FACS (Fig. 2B). Then we investigated the expression of TLRs in MSCs (Fig. 2C). Our results showed that rat MSCs express TLR1, 2, 3, 4, 6, and 9 mRNA (Fig. 2C).

Homing of MSCs in Partially Hepatectomized Rats

We partially hepatectomized rats and then injected CM-DiI labeled MSCs isolated from normal animal's bone marrow (Fig. 3). No injection of MSCs was our negative group (Fig. 3A-B). CM-DiI labeled MSCs were administered to the animal that did not undergo PH (Fig. 3C-D), to 1 day post-PH group (Fig. 3E-F), to 3 days post-PH group (Fig. 3G-H) and to 5 days post-PH group (Fig. 3I-J) through their tail vein. Three days after MSC administration, animals were sacrificed. Fluorescence microscopy studies revealed that a thin layer of CM-DiI positive MSCs were localized at the outermost border of the PH liver sections only in 3 days post-PH animals (Fig. 3H). Untreated, 1 day and 5 days post-PH animals gave no detectable CM-DiI specific signal (Fig. 3D, 3F and 3J respectively). As expected, no staining in the liver sections was observed when MSCs were not injected (Fig. 3B). This data implicated that MSCs were accumulating in the liver upon liver injury in a specific timing (Fig. 3H).

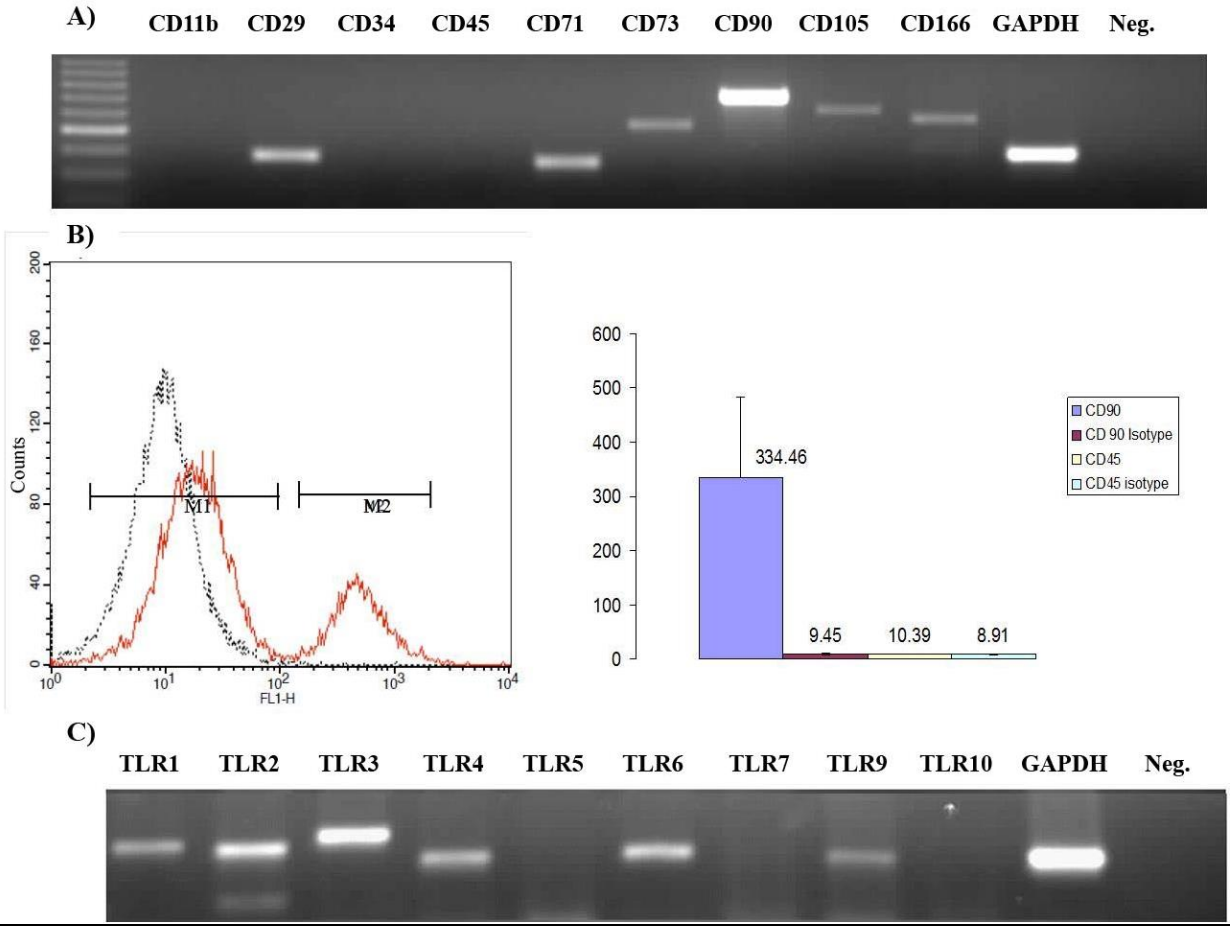

Fig. 2. Characterization and TLR expression profile of MSCs. A) Gel picture showing MSC-specific positive (CD 29, CD71, CD73, CD90, CD105 and CD 106) and negative (CD 11b, CD34 and CD45) marker transcripts, B) Quantification of CD90 and CD45 proteins expressed by MSCs (MSCs were positive for CD90 (84.5±7.2 % of total population, MFI: 334.5±150.3) and negative for CD45 (96.6±1.2 % of total population, MFI: 10.4±0.5)) by FACS (at day14), C) PCR gel image showing the MSC expression levels of rat (TLR1, 2, 3, 4, 6, and 9 mRNA) amongst TLR1-10.

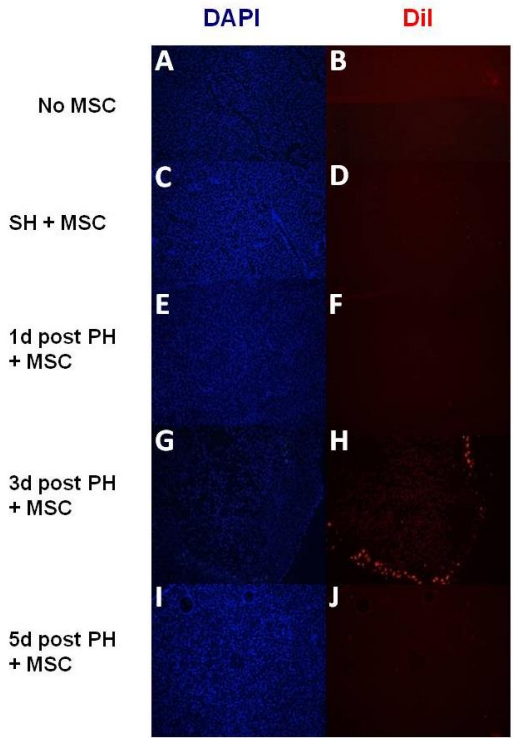

Fig. 3. Fluorescence photomicrographs of the liver sections injected with CM-DiI labeled MSCs. CM-DiI labeled MSCs were injected into SH (C, D), 1 day post-PH (E, F) 3 days post-PH (G, H) and 5 days post-PH (I, J) rats. Background staining from untreated and no MSC received liver sections (A, B). Thin layer of CM-DiI positive MSCs were localized at the outermost border of the PH liver sections only in 3 days post-PH animals (H). Arrows: CM-DiI labeled MSCs. Magnification: 20X.

CD90 Expression in Partially Hepatectomized Livers

After observing the presence of CM-DiI positive MSCs in PH liver, contribution of resident MSCs mediating liver regeneration process in the injured niche was examined. We performed immune staining for CD90 to investigate the recruitment of resident MSCs adjacent to CM-DiI labeled allogeneic MSCs (Fig. 4). The presence of the cells was shown by DAPI staining (Fig. 4A). The accumulation of the injected MSCs was evident by CM-DiI staining (Fig. 4B). Several resident CD90 positive MSCs (Fig. 4C) were present and scattered around CM-DiI-labeled MSCs as evidenced by merging of CM-DiI and FITC-CD90 (Fig. 4D) in the liver section of the 3 days post-PH rats. Consistent with earlier observations, no specific CD90 staining was observed for normal, 1 day and 5 days post-PH rats (data not shown).

FLT3 Expression in Partially Hepatectomized Livers

Oval cells are known to play pivotal role during progenitor-dependent liver regeneration and they express FLT3 on their cell surface (Alison 1998). Immunofluorescent staining against FLT3 was performed to check the existence of hepatic oval cells in the hepatectomized liver sections after the administration of CM-DiI-labeled MSCs (Fig. 5). FLT3 positivity was observed in the vicinity of CM-DiI positive MSCs in the liver of 3 days post-PH rats (Fig. 5A-D).

TLR Expression Following MSC Infusion During Liver Regeneration

In order to understand the contribution of TLRs during homing process of MSCs in liver regeneration, the changes of the expression patterns of several TLRs from normal and hepatectomized livers over 24 h by real-time RT-PCR were examined. From C*t* values, fold change in expression for each TLR was plotted (Fig. 6A-F). From these plots, it was apparent that specifically four genes (TLR2, TLR3, TLR5 and TLR 9) remained unaltered (i.e. expression levels ~ 1.0) over the course of first 24 h post-PH compared to normal liver (Fig. 6A, 6B, 6D and 6F). Before investigating further, we omitted the potential contribution of TLR 5 due to the fact that MSCs do not express this mRNA (Fig. 2C).

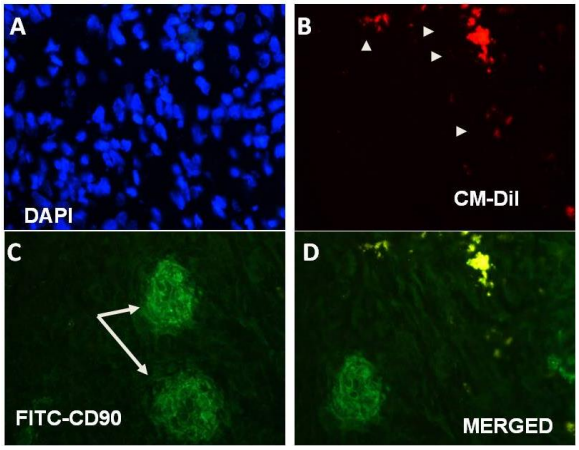

Fig. 4. Immunofluorescence photomicrographs showing CD90 expression. Sections were from 3 days post PH liver that had been injected with CM-DiI labeled MSCs revealing the contribution of resident MSCs mediating liver regeneration process in the injured niche. (A) DAPI, (B) CM-DiI (C) CD90-FITC (D) merged. Arrows: CD90+ areas. Arrow Heads: CM-DiI positive areas. Magnification: 20X.

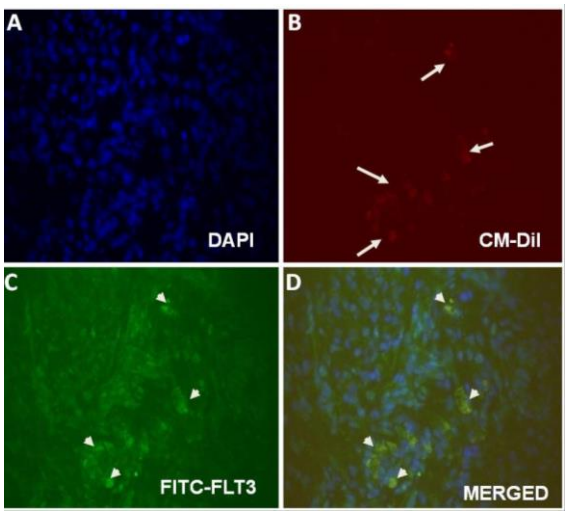

Fig. 5. Immunofluorescence photomicrograph showing FLT3 expression. Sections were from 3 days post PH liver that had been injected with CM-DiI labeled MSCs revealing the existence of hepatic oval cells in the hepatectomized liver sections. (A) DAPI (B) CM-DiI (C) FLT3-FITC and (D) Merged. Arrows: CM-DiI and arrow heads: FITC-FLT3 positive areas. Magnification: 20X.

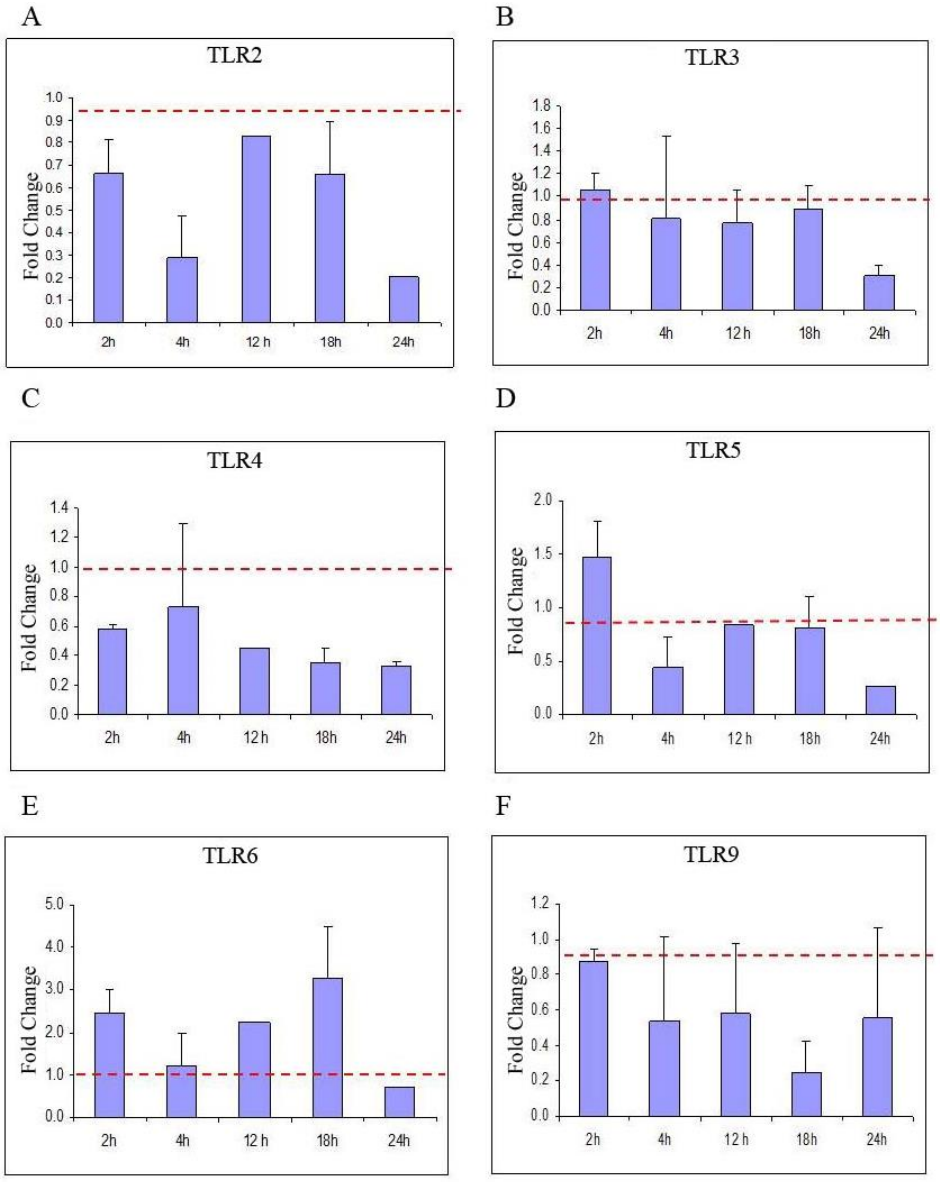

Fig. 6. qRT-PCR profiles showing time course fold change of (A) TLR2, (B) TLR3, (C) TLR4, (D) TLR5, (E) TLR6 and (F) TLR9 gene transcript levels following PH over 24 h. TLR2, TLR 3, TLR 5 and TLR 9 remained unaltered over the course of first 24 h post-PH compared to normal liver.

Next, we checked changes in TLR expression level upon MSC administration during liver regeneration. To our knowledge, no study has attempted to delineate the changes in TLR expression and MSC accumulation following injury. In order to differentiate whether TLR2, TLR3 and TLR9 are involved in the homing of administered MSCs to the injured site, the time-course fold change in their transcript levels was investigated. Our results revealed that TLR2, TLR3 and TLR9 mRNA expression levels increased in liver samples of partially hepatectomized rats that received labeled MSC in comparison to those animals that did not have any MSC treatment (Table 3). Following MSC injection, TLR2 and TLR9 mRNA levels were significantly higher than TLR3 in regenerating liver (ca. 7 fold for TLR2 and 9 vs ~2 fold for TLR3).

Table 3. Fold change in TLR2, TLR3 and TLR9 expression of PH animals before and after MSC administration from normal rats.

Rx groups	TLR2	TLR3	TLR9
Mock + MSC	1.9 ± 0.3	2.2 ± 0.5	2.8 ± 0.1
PH (1d)	1.3 ± 0.2	0.3 ± 0.1	1.6 ± 0.6
PH (1d) + MSC	5.7 ± 1.1	1.7 ± 0.4	3.9 ± 0.7
PH (3d)	1.3 ± 0.3	0.6 ± 0.2	1.2 ± 0.4
PH (3d) + MSC	7.7 ± 0.9	2.1 ± 0.1	4.3 ± 0.5

Discussion

In this study, we aimed to understand the immune response of liver upon BM derived MSCs infusion through changes in TLR expression of remaining liver and delineate their contribution to regeneration process.

There are several heartening therapeutical applications of MSCs in osteogenesis imperfecta, hematopoietic recovery, bone tissue regeneration, cardiovascular repair, spinal cord injury, coronary artery disease and also in several organ failures such as lung fibrosis in animal models (Horwitz *et al.* 1999, Koc *et al.* 2000, Petite *et al.* 2000, Minguell *et al.* 2001, Matty 2008). Although Popp *et al.* (2007) reported that multipotent mesenchymal stromal cells do not differentiate into hepatocytes *in vivo* when transplanted in regenerative conditions, Lee *et al.* (2004) showed that bone marrow-derived MCS from human differentiate into functional hepatocyte-like cells under defined conditions pointing out the potential for clinical relevance. Moreover, recent findings suggested that these cells can effectively rescue experimental liver failure (upon CCl_4 administration) and contribute to liver regeneration. Collectively, accumulating data implicated that MSCs are suitable cell-based tools as an alternative therapy to organ transplantation for the treatment of liver diseases (Kuo *et al.* 2008). The signals driving MSCs to the site of injury during healing is not convincingly resolved and there are conflicting reports describing the physiologic roles of TLR expression on isolated/generated MSCs. These controversies prompted us to investigate in detail the relevance between migration of MSC to and contribution of TLR*s* to this process using hepatectomized rat as a model in experimental liver regeneration.

In order to chase homing of MSCs in liver after PH, CM-DiI labeled MSCs from normal rats were administrated into the 1, 3 and 5 post-PH rats. Our results revealed that only in 3 day post-PH rat, MSCs were localized in the liver (Fig. 3H).

Next, we asked the question of whether endogenous MSCs also show the similar homing pattern to allogeneic MSCs during liver regeneration in terms of timing and localization. Our *immunoflourescein* data for CD90 staining in the liver sections revealed that the time of the appearance of endogenous MSCs are synchronous to that of exogenously localized allogeneic MSCs. These cell types were co-localized at the liver regeneration site 3 days post-PH (Fig. 4C and 4D). This suggested that either localized administered MSCs induced further accumulation of the syngeneic MSCs, or the accumulation process of the resident MSCs reached to a plateau, and furthermore initiated injected MSCs to localize at the injury site. Further studies to clarify these alternatives are required.

Oval cells are playing pivotal role in progenitor-dependent liver regeneration and are known to express specific proteins at their surfaces (Allison 1998). Among these, FLT3 is one of the candidate proteins (Agnes *et al.* 1994). FLT3 is a receptor tyrosine kinase **(**RTK) and a well-known hematopoietic stem cell marker (Agnes *et al.* 1994). We previously showed the expression of FLT3 both at mRNA and protein level during progenitor cell-dependent liver regeneration (Aydin *et al.* 2007). In this study, FLT3 expression was observed only when MSCs administrated into 3 days PH rats in the vicinity of labeled MSCs. Expression of FLT3 around this location suggests the onset of progenitor-dependent liver regeneration. However, we do not know the factors that regulate the specific pattern of MSC localization in the regenerated liver. Future studies to elucidate these factors (such as chemo attractant molecules) are expected to better understand liver regeneration process and provide very useful information for possible therapies.

Faust *et al.* (2006) proposed that three major pathways may regulate the circuitry required for liver regeneration. These are i) cytokine, ii) growth factor and iii) metabolic networks linking liver function to cell growth and proliferation. It is proposed that the innate immune system plays an important role in the initiation of liver regeneration after partial hepatectomy (Hritz *et al.* 2008). In particular, IL6 and TNFα production by Kupffer cells are found to be required for initiation of liver regeneration after PH, although the activation processes are still unknown. TLRs are important upstream elements of proinflammatory cytokine networks. Watanabe *et al.* (2007) showed that IL6 and TNFα production decrease significantly leading to defective liver regeneration in $MyD88^{-/-}$ mice after PH. However, it was also shown that TLR 2, 4 and 9 are not essential for NF-κB activation and IL6 secretion (Seki *et al.* 2005, Campbell *et al.* 2006). The latter observation is particularly surprising since enteric-derived LPS (ligand of TLR4) was shown as the stimulating agent for proinflammatory cytokine production at the start of liver regeneration (Cornell 1985, Cornell 1990, Shiratori *et al.* 1996). Therefore, we decided to investigate the mRNA expression of several *TLR* genes in 0 h, 2 h, 4 h, 12 h, 18 h and 24 h after PH (Fig. 6). Our results revealed that induction in the expression of TLR 2, 3, 5 and 9 remain nearly unchanged during this testing period compared to the expression levels of normal animals at 0h.

We hypothesized that the mechanism of MSC homing to injury site may be due to TLR expression of the organ in which injury occurs, and investigated the relationship between TLR expression profiles of hepatectomized liver upon MSC infusion. It was shown that TLR stimulation on human MSC drives their migration *in vitro* (Tomchuck *et al.* 2008). In the light of this data, one can hypothesize that certain TLR expression at the injured tissue/organ may be critical for the MSC migration leading to regenerative process. Our results strongly suggest the involvement of TLR 2, 3 and 9 due to following observations: i) their expressions are relatively unchanged over the period of 24 hours after PH of the host animals in the absence of MSC infusion (Fig. 6), ii) mRNA expression levels of TLR 2, 3 and 9 are increased in MSC-administrated 1day PH and 3days PH rat liver samples in comparison to that of PH animals that had no MSC injection (Table 1). Thus, the injected MSCs induced the alteration of TLR 2, 3 and 9 levels in the remaining liver during regeneration.

In conclusion, our data suggested that the driving force of MSC homing is not dependent on TLR expression of MSCs, rather TLR upregulation in the micro environment of the injured liver in part may dictate MSC homing. Thus, immune response mediated by TLR signaling during regeneration process is critical for MSC homing.

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Review

STEM CELL TREATMENT IN DEGENERATIVE RETINAL AND OPTIC NERVE DISEASES

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Abstract: Use of stem cells in the treatment of retinal diseases is a new and popular topic in ophthalmology. Embryonic and bone marrow derived stem cells can be used for treatment. Age-related macular degeneration, Stargardt's macular dystrophy and retinitis pigmentosa are common encountered retinal diseases causing progressive vision loss. The researches therefore mostly focus on these diseases which have no curative treatment modality in order to evaluate the efficacy of stem cell therapy. In this review, we aimed to present the results of the phase 1/2 clinical studies about stem cell treatments in eye diseases. Stem cell therapies are the rising trends in treatment of retinal diseases. Further clinical studies are required for standardization of the therapy and obtaining long-term data about the results and complications.

Key words: Optic nerve diseases, retinal diseases, stem cell therapy.

Özet: Retina hastalıklarının tedavisinde kök hücrelerin kullanımı oftalmolojide yeni ve popüler bir konudur. Embriyonik ve kemik iliği kaynaklı kök hücreler tedavi için kullanılabilir. Yaşla bağlı maküla dejenerasyonu, Stargardt'ın maküla distrofisi ve retinitis pigmentosa, progresif görme kaybına neden olan yaygın görülen retina hastalıklarıdır. Bu nedenle araştırmalar kök hücre tedavisinin etkinliğini değerlendirmek için herhangi bir küratif tedavi modalitesine sahip olmayan bu hastalıklara odaklanmaktadır. Bu yayında göz hastalıklarında kök hücre tedavisi ile ilgili faz 1/2 klinik çalışmaların sonuçlarını incelemeyi amaçladık. Kök hücre terapileri, retinal hastalıkların tedavisinde yükselen bir trenddir ve tedavinin standardizasyonu, sonuçlar hakkında uzun vadeli veriler elde etmek ve komplikasyonlara ilişkin daha ileri klinik çalışmalara ihtiyaç vardır.

Introduction

Degenerative retinal diseases are among the main causes of irreversible vision loss. Most of these degenerative processes affect the outer retina, which include photoreceptors and retinal pigment epithelium (RPE). Most retinal diseases occur due to apoptosis of retinal neural cells or adjacent supporting tissue. The underlying mechanism of most diseases can be different but retinal degeneration is the end point (Strauss 2005). Although there is no effective treatment for these degenerative diseases, stem cell implantation, following the succesful results of experimental studies in recent years, is now available as a treatment option to restore visual function in these diseases (Lamba *et al.* 2009).

Type of Stem Cells in Ophthalmology

Stem cell is defined as a pluripotent or multipotent cell, which has the ability of self renewal and differentiation. These cells are capable of differentiating into other cell types of the body. They also have the potential to repair tissue and restore function after injury (Siqueira *et al.* 2010, Siqueira 2011). Adult stem cells, induced pluripotent stem cells (IPSCs) and mesenchymal stem cells (MSCs) are non-embriyonic stem cells and have been widely used in the treatment of retinal diseases (Zarbin 2016).

Embryonic stem cells (ESCs) are special pluripotent cells derived from internal cell mass of the embryo (blastocyst). These cells have the capability to differentiate into other types of body cells. Although they are valuable in the treatment of transplantation in some diseases, their use is limited for various reasons. The process can lead to destroy or disaggregate embryos so it still continues to cause ethical concerns and is prohibited by law in many countries. However some studies showed that it is possible to obtain these cells without destroying the embryo (Chung *et al.* 2008).

Mesenchymal stem cells may be derived from many tissues such as bone marrow, fat, skin and cartilage. Bone marrow MSCs (BMMSCs) and adipose-derived MSCs (ADMSCs) are the most common type of stem cells that are used as a source of different retinal cells. These cells

are considered as multipotent cells, which can differentiate into many types of specialized cells in the body (Yu *et al.* 2007)

Induced pluripotent stem cells are pluripotent like ESCs and they can differentiate into other types of retinal cells. The process of IPSC producution for therapy has a high cost and is achieved by application of long protocols (Alonso-Alonso & Srivastava 2015)

Cord blood stem cells are isolated in vitro from cord blood following delivery. Amniotic fluid stem cells are isolated in vitro from cells obtained from amniotic fluid (Zarbin 2016).

In recent years, there have been significant developments about stem cells. MSCs have the advantages of trophic support that allows slowing down of retinal cell degeneration and immunosuppression, but, on the other hand, they have low rates of cell migration and differentiation. Compared to BMMSCs, ADMSCs have the advantages of easier harvest from donors, faster expansion, more protein secretion and higher immunomodulatory capacity. MCSs increase support to surrounding cells by secreting growth factors, show anti-apoptotic, anti-inflammatory, immunomodulatory and angiogenic effects. It is thought that various cytokines, growth factors and proteins, which are secreted by stem cells, can cause photoreceptor survival promotion. Thus, it could be useful to treat retinal diseases in their early stage (Jones *et al.* 2017, Tang *et al.* 2017, Öner 2018).

There are a number of advantages of stem cell applications in the eye. The amount of required stem cells is low which reduces the cost. The surgical technical is easy and the transplanted cells can be seen with the imaging methods used in clinical practice. The non-operated eye can be used as a control to evaluate the effect of the therapy. The eye is known as immun priviliged and long-term immunosuppressive treatment is not required after the implantation. The application of healthy stem cells in the place of degenerated retinal cells has promoted cell regeneration, creation of new intercellular connections, and improvement of visual function. Stem cells have the potential to differentiate into many cells in their environment, including the retinal neural cells and photoreceptors. Earlier experimental studies have shown that stem cells are very compatible with the retina and are able to adapt to Müller, amacrine, bipolar, horizontal and glial cells, and photoreceptors (Tucker *et al.* 2014, Whiting *et al.* 2015).

Retinal degeneration occurs in several forms, such as age-related macular degeneration (AMD), Stargardt's macular dystrophy (SMD) and retinitis pigmentosa (RP).

Studies on Retinitis Pigmentosa

Retinitis pigmentosa is the most common form of inherited progressive retinal dystrophy. It mainly affects rod photoreceptors and gives rise to progressive loss of rod and cone photoreceptors than RPE cells. This initially manifests as nyctalopia or difficulty with night vision which is the initial manifestation of impairment of the photopigment retinol function and cycling. Night blindness and progressive visual field loss, often leading to complete blindness can be seen in different stages of the disease, and no curative treatment has been described yet (Rivolta *et al.* 2002, Hims *et al.* 2003, Ramsden *et al.* 2013).

A clinical study was performed with 20 RP patients by using intravitreal bone-marrow-derived stem cells and the quality of life was determined with National Eye Institute Visual Function Questionnaire-25 (NEI-VFQ) 3 months after the injection. The results showed that the quality of life of the patients improved significantly 3 months after the treatment, but by the 12^{th} month there was no significant difference from baseline (Siqueira *et al.* 2015). It was reported that functional visual score, functional field score, and functional visual acuity of the American Medical Association guidelines were equally correlated to the questionnaire in the same RP patients. No adverse effects were reported (Seo *et al.* 2009).

Parks *et al.* (2015) reported the findings of a phase 1 clinical trial of intravitreal autologous bone marrow $CD34^+$ cell injection. Various ischemic and degenerative retinal disorders were included in the study. Intraocular inflammation or hyperproliferation were not observed during the study. Their findings showed that the treatment was safe and a patient with RP was noted to have improvement on visual function on Goldmann perimetry which appeared sustained at the 6-month follow-up visit.

In a recent study, the safety of a single intravitreal injection of autologous BMMSCs in patients with advanced RP was studied (Satarian *et al.* 2017). Stem cells were also injected into the mouse vitreous cavity for further evaluation. No adverse events were observed in eyes of 2 out of 3 patients who reported improvement in light perception which lasted for 3 months. The third patient experienced extensive pre-retinal and vitreal fibrosis starting at the second week of the therapy and further ciliary injection, cyclitic membrane, shallow anterior chamber, ocular hypotony and nearly total tractional retinal detachment at the 3 month follow-up. Ocular examination revealed vision of no light perception, mature cataract, iris neovascularization, ocular hypotony and shallow anterior chamber at 1 year follow-up.

In a phase 1 clinical safety study, subretinal ADMSC implantation was performed to advanced stage RP patients. There was no statistically significant difference in visual acuity from baseline. Four patients experienced visual acuity improvement during the 6 month follow-up. Although there were no systemic complications, 5 of the 11 patients experienced various ocular complications. One patient had choroidal neovascularization (CNV) at the site of the implantation which was treated with one injection of intravitreal anti-VEGF. Five patients had ERM around the transplantation site and at the periphery causing localized peripheral tractional retinal detachment at the periphery (Öner *et al.* 2016).

Recently, seventeen patients with bilateral visual loss due to RP included Stem Cell Ophthalmology Treatment Study (SCOTS and SCOTS 2) and followed-up at least 6 months. Affected eyes were treated with autologous BMMSCs. Patients were treated either retrobulbar, subtenon and intravenous SCs or retrobulbar, subtenon, intravitreal and intravenous. In 33 treated eyes, 15 eyes (45.5%) improved an average of 7.9 lines of Snellen acuity, 15 eyes (45.5%) remained stable, and 3 eyes (9%) worsened by an average of 1.7 lines of Snellen acuity. No surgical complications were reported. The study also reported that duration of the disease did not appear to affect the ability of the eyes to respond (Weiss & Levy 2018)

Studies on Aged-related Macular Degeneration and Stargardts' Macular Dystrophy

Age-related macular degeneration is one of the causes of vision loss in developed countries. It affects elderly patients with progressive and irreversible deterioration of central vision. The disease prevalence is expected to increase in the near future (Wong *et al.* 2014). In contrast to AMD, SMD affects mainly young people, often starting in late teens or early 20s. It is the most common inherited macular dystrophy in both adults and children. Similar to AMD, central visual loss is seen. These patients have also dyschromatopsia and central scotoma with characteristic macular atrophy and yellow white flecks at the level of the RPE (Fujinami *et al.* 2013, 2015).

Schwartz *et al.* (2012) studied with human ESCs (hESCs) for the first time in AMD and SMD. The results of subretinal transplantation of hESC-derived RPE in patients with SMD and the dry type AMD revealed no hyperproliferation, abnormal growth or immune mediated transplant rejection during the first 4-month period. hESC-derived cells were well tolerated for up to 37 months without serious adverse effect. Visual acuity improved in ten eyes, improved or remained the same in 7 eyes and decreased by more than ten letters in one eye, whereas the untreated fellow eyes of the patients did not show similar improvements. The results of this study provided the first evidence of the medium-term to long-term safety, graft survival and possible biological activity of pluripotent stem cells in individuals with retinal disease (Schwartz *et al.* 2015).

Another study about the safety and tolerability of subretinal transplantation of hESC-derived RPE was published by Song *et al.* (2015). The study consisted of 4 patients, 2 with AMD and 2 with SMD. Improvement of 9-19 letters in visual acuity in 3 patients was identified after 1-year follow-up. The other patient with AMD remained stable. One patient with AMD developed an epiretinal membrane (ERM) persisting at 1-year follow-up with retinal puckering and choroidal neovascularization (CNV) and was treated with intravitreal anti-VEGF. The other patient with AMD also developed ERM's, intraretinal cysts and dye pooling on fluorescein angiography persisting throughout the period of the study. Song *et al.* (2015) concluded that the long-term safety and efficacy of hESC-derived RPE required further studies.

In the study of Limoli *et al.* (2014), the patients received a cell graft between choroid and sclera. The researchers used ADMSCs in 12 eyes of 12 patients with AMD. In addition to these stem cells, platelet-rich plasma (PRP) was also used. Electrophysiological evaluation of all eyes enrolled in the study revealed a significant increase especially in the electoretinogram (ERG) values recorded by scotopic rod-ERG after cellular autograft. No adverse effects were reported in any of the patients.

Limoli *et al.* (2016) used the same technique in another study with a larger group of patients. 36 eyes of 25 patients with AMD received implantation of ADMSCs and platelets from PRP in the suprachoroidal space. After 6 months, the treatment improved visual performance and the increase was better if retinal thickness recorded by OCT (optic cohorens tomography) was higher. They assumed that a greater number of residual cells lead to greater interaction between growth factors and chorioretinal cellular membrane receptors, more intense cellular activity and, ultimately, improvement of visual quality.

In a recent case report, subretinal transplantation of autologous iPSC-derived retinal cells generated from skin fibroblasts in a patient with neovascular AMD was described (Mandai *et al.* 2017). The removal of the neovascular membrane was performed at the surgery. After 1-year-follow up, the patient remained stable in terms of visual acuity still having cystoid macular edema. There was no sign of graft rejection or recurrence of the neovascular membrane. IPSC-based autologous transplantation was reported to be safe and feasible in the treatment of the patient (Mandai *et al.* 2017).

Kuriyan *et al.* (2017) reported 3 patients with AMD with the development of bilateral severe visual loss after receiving intravitreal injections of autologous ADMSCs at stem-cell clinics associated with ocular hypertension, hemorrhagic retinopathy, vitreous hemorrhage, combined tractional and rhegmatogenous retinal detachment or lens dislocation.

BMMSCs were used intravitreally in the treatment of 60 advanced dry AMD patients in another study (Kumar *et al*. 2017). The effect of stem cell therapy was evaluated in terms of visual acuity, amplitude and implicit time in multifocal-ERG (mf-ER) and the size of geographic atrophy. Although no statistically significant improvement in the best corrected visual acuity (BCVA) after 6 month follow-up occurred, mf-ERG showed significant improvement in amplitude and implicit time in the treated group. Adverse events were not seen in any of the patients. The researchers concluded that the electrophysiological and anatomical improvement in the treatment group may indicate the therapeutic role of BMMSCs in patients with dry AMD (Kumar *et al*. 2017).

The safety and efficacy of intravitreal injections of bone marrow mononuclear fraction (BMMF) containing $CD34^+$ cells in 10 patients with atrophic AMD were studied (Cotrim *et al.* 2017). Patients were evaluated with the tests including microperimetry, infrared imaging, fundus fluorescein angiography (FFA) and OCT. During the 12-month follow-up, mean BCVA and mean sensitivity threshold improved significantly. Patients who have smallest areas of atrophy had better results. Choroidal new vessels or tumor growth were not identified according to FFA tests. The authors emphasized that the paracrine effect of $CD34^+$ cells may explain the functional improvement observed in their study (Cotrim *et al.* 2017).

Another prospective clinical case series aimed to investigate the safety and efficacy of suprachoroidal ADMSC implantation in 4 patients with Dry AMD and 4 patients with SMD. All of the patients experienced visual acuity, visual field and mf-ERG recording improvement during the 6 month follow-up whereas the untreated fellow eyes of the patients did not show similar improvements. No systemic or ocular complications were found during the 6 month follow-up. Stem cell treatment with suprachoroidal implantation of ADMSCs seems to be safe and effective in the treatment of dry type AMD and SMD (Oner *et al.* 2018).

Studies on Optic Neuropathies

In the study of Weiss *et al.* (2017), 10 patients with bilateral visual loss due to sequential non-arteritic ischemic optic neuropathy (NAION) underwent autologous BMMS therapy within the Stem Cell Ophthalmology Treatment Study (SCOTS). Affected eyes were treated with either retrobulbar, subtenon and intravenous stem cells or following vitrectomy, intra-optic nerve, subtenon and intravenous stem cell applications. Following the therapy in SCOTS, 80% of the patients experienced improvement in Snellen binocular vision (P=0.029) with 20% remaining stable, and 73,6% of the eyes treated gained vision (P=0.019) and 15,9% remained stable in the post-operative period. Improvements typically manifested no later than 6 months post procedure. Duration of the visual loss did not appear to affect the ability of the eyes to respond to the treatment. Statistically significant improvements in the visual acuity of individual eyes and of binocular vision in this condition have been shown (Weiss *et al.* 2017).

In a case report, a relapsing auto-immune optic neuropathy caused progressive bilateral visual loss, and the patient underwent vitrectomy with intra-optic nerve injection of BMMSCs in the right eye and retrobulbar, subtenon and intravitreal injection of BMMSCs in the left eye. Both eyes continued to have visual field improvements 1 year after SCOTS treatment. 3 and 6 months after SCOTS treatment, both macular thickness maps and fast retinal nerve fiber layer thickness improved (Weiss *et al.* 2015).

In 5 Leber's herediter optic neuropathy patients who underwent SCOTS treatment, improvements in visual acuity and peripheral vision were found. Several of the eyes experienced dramatic, persistent increases in visual acuity attributable to the BMMSC treatment in SCOTS including finger count to 20/100 and hand motion to 20/200. Visual field improvements were noted. Macular thickness and optic nerve head thickness varied and did not appear to be correlated with vision improvements. No adverse or serious adverse events were observed. These improvements could be a result of revitalization of existing mitochondrial function and the transfer of more viable mitochondria in existing neurons and glial cells, as well as transdifferentiation of the BMMSCs and incorporation of newly developed cells in the existing ganglion and optic nerve cell layers. Further exploration of stem cell treatment in mitochondrial disease appears warranted (Weiss *et al.* 2016).

Conclusion

Stem cell treatment modalities are the rising trends in retinal disease. The common diseases causing vision loss including AMD, SMD and RP have some promising results when treated with ESC or MSCs. Also, there are continuing studies, many ongoing clinical trials aimed to evaluate the stem cell treatment in retina and optic nerve diseases. Today, there is no standardization for this therapy. Long-term safety and efficacy of stem cell application should be followed. More clinical trials should evaluate the methods, timing of the applications and follow-up results. In the near future, the regenerative stem cell therapy may be a standard treatment modality in many degenerative eye disorders.

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Review

HEMATOPOIETIC STEM CELL GENE THERAPY FOR INHERITED MONOGENIC DISEASES AND ITS IMPLICATIONS FOR FUTURE GENE THERAPY TRIALS IN TURKEY

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Abstract: Stem cell therapy offers a great advantage for the development of new treatments in the field of regenerative and restorative medicine. However, the use of stem cell therapies and their clinical indications can even be further improved using genetic modification of the cells. Due to the high level of consanguineous marriages in Turkey, the country suffers from an increased frequency of inborn genetically inherited diseases. Treatment of these diseases is difficult, since 1) diagnosis is often delayed in rural areas, 2) distance to specialized centers may be considerable, 3) treatment may require frequent hospital visits and 4) treatment procedures are often both invasive and expensive.

Here, we discuss the current status of gene therapy of hematopoietic stem cells (HSCs) for rare, inherited monogenic diseases and the advantages to use these cells as an alternative treatment option for patients in Turkey. We discuss results of clinical trials using retroviral and lentiviral gene therapy for the treatment of immune deficiencies, hemoglobinopathies and several enzyme deficiencies, new developments in the field of the HSC gene therapy to improve safety and efficacy and recommendations for the future.

Key words: Gene Therapy, hematopoietic stem cells, lentiviral vectors, biosafety, inherited monogenic diseases.

Özet: Kök hücre tedavisi rejeneratif tıp ve restoratif tıp alanlarında yeni tedavilerin geliştirilmesi için büyük avantaj sağlamaktadır. Böylelikle, kök hücre tedavilerinin kullanımı ve bunların klinik endikasyonları hücrelerin genetik modifikasyonu ile geliştirilebilmektedir. Türkiye'deki akraba evlilik oranının fazla olmasından dolayı, yenidoğan genetik kalıtsal hastalıkların insidası artmaktadır ve bu durum bir sorun teşkil etmektedir. Bu hastalıkların tedavi edilmesi; 1) kırsal bölgelerde hastalığın sıklıkla geç teşhisi, 2) Özel merkezlerin uzak olması, 3) Tedavilerin sık hastane kontrolü gerektirmesi, 4) Tedavi prosedürlerinin hem pahalı hem de invazif olması nedenleriyle zordur.

Bu makalede nadir kalıtsal monogenik hastalıklar için hematopoetik kök hücre (HKH) gen tedavisinin güncel durumları ve Türkiye'deki hastalar için alternatif bir tedavi seçeneği olarak kullanımının avantajları tartışılacaktır. İmmun yetmezlikler, hemoglobinopatiler, birçok enzim eksikliklerinde retroviral ve lentiviral gen tedavi klinik çalışma sonuçları, HKH gen tedavisi alanındaki yeni gelişmeler, güvenlik ve etkinliğin artırılması ve gelecekteki öneriler tartışılacaktır.

Introduction

Current treatment strategies for inherited monogenic diseases

The rationale for stem cell gene therapy for inherited diseases is to provide the genome with a healthy copy of the gene as an addition to or as a replacement of the mutated gene in order to develop permanently curative treatment options for inherited monogenic diseases. Many hematopoietic diseases and enzyme deficiencies can be currently treated with hematopoietic stem cell (HSC) transplantation (Biffi 2017, Majhail *et al.* 2015, Ringden *et al.* 2018, Wynn 2011). In addition, Enzyme Replacement Therapy (ERT) has been developed for some of the enzyme deficiencies/storage diseases, such as Fabry disease, Gaucher disease, Pompe disease, Lysosomal acid lipase deficiency, Mucopolysaccharidosis (MPS-I: Hurler Syndrome; MPS-II: Hunter Syndrome; MPS-IV: Morquio Syndrome; MPS-VI: Maroteaux–Lamy syndrome), as well as for severe combined immunodeficiency (SCID) caused by adenosine deaminase deficiency (ADA-SCID) (Beck 2018, Gaspar *et al.* 2009). However, not all patients have an available matched donor, and even if a donor is available, the HSC transplantation will fail when

expression levels of the enzyme in HSCs are too low to expect benefit, or may result in graft-versus-host-disease (GvHD) when cells derived from the donor induce immunological damage of tissues/organs of the patient. In addition, ERT often involves life-long weekly-monthly treatments requiring frequent (out-patient clinic) hospital admissions and effects of ERT typically decline in time due to immune responses against the drugs, resulting in even more frequently needed infusions. Moreover, the inability of enzymes to efficiently cross the blood-brain barrier (BBB) limits the efficacy of both HSC transplantation and ERT in enzyme deficiencies that affect the central nervous system (CNS).

Development of novel treatments for rare diseases

Rare or orphan diseases are diseases which affect fewer than 200,000 people (US definition) or less than 5 individuals per 10,000 of the population (EU definition) and as a result are not the focus of interest of the pharmaceutical industry because of the little financial incentive to develop new medications (Sharma *et al.* 2010). Approximately 70% of about 6,000-8,000 diseases which are considered rare are genetic in their origins. To encourage pharmaceutical companies to invest in orphan drug development, the US introduced the US Orphan Drug Act in 1983 to provide suitable tax initiatives on clinical trials and 7 years of marketing exclusivity for drugs developed for conditions that occur only rarely in the US (Shah 2006). Since then, over 250 orphan drugs have been approved by the US Food and Drug Administration (FDA) and in its footsteps, a number of other countries introduced similar legislation (Singapore in 1991, Japan in 1993 and Australia in 1998) (Shah 2006). In 2000, the European Parliament and Council Regulation (EC) adopted the orphan medicinal products legislation with regulations No 141/2000 (EC No 141/2000) and No 847/2000 (EC No 847/2000). The EMEA (now EMA), through its Committee for orphan medicinal products (COMP) is responsible for reviewing designation applications. During the first five years of implementation (from April 2000 to April 2005), 458 applications for orphan designation were submitted, resulting in 268 products being designated for more than 200 different rare conditions (EC No 141/2000). The predominant therapeutic areas covered were cancer (36%), metabolic disorders (21%), immunology (11%), and cardiovascular and respiratory disorders (12%) (EC No 141/2000). Of these, 54% of the medicinal products designated had potential for pediatric use, 11% solely for pediatric and 43% for both adult and pediatric use (EC No 141/2000). Unfortunately, research funds barely cover the costs of the scientific community and development of new innovative medicinal products is expensive. It is estimated that the average cost of developing a new drug ranges from 400 to 800 million USD depending on the therapeutic class of the drug (DiMasi *et al.* 2003). Commercially, the costs for the development of a drug for an orphan disease often do not outweigh the expected returns. As a result, prices may be high and access may vary from country to country and depend on the availability of health insurance or governemental reimbursements.

Gene therapy for inherited monogenic diseases

After an initial period of low success rates with unforeseen severe adverse effects and poor public understanding resulted in the (temporarily) seizing of ongoing clinical trials, gene therapy is now becoming more and more attractive as a treatment option for rare, inherited monogenic diseases. Not only because the treatment is (intended to be) curative and a single treatment should be sufficient, but also because the costs of a single treatment would be significantly lower than the costs of life-long symptomatic treatment. The use of genetically modified autologous HSCs to treat monogenic diseases is based on the assumption that 1) the disease itself can be treated by the transfer of healthy HSCs because the hematopoietic stem cell and/or its progeny are affected, e.g. primary immune deficiencies (PID) or diseases involving hemoglobin synthesis (hemoglobinopathy), 2) addition of a single normal copy of the mutated gene is sufficient to correct the deficiency or alleviate its symptoms or 3) overexpression of the gene in hematopoietic cells results in sufficient levels of the missing protein, resulting in cross-correction of affected cells and clearance of the accumulated substrate, e.g. enzyme deficiencies. In addition, gene therapy in mitotic cells, such as HSCs, lymphoid progenitor cells and mature lymphocytes requires the use of a vector system such as retrovirus or lentivirus which are able to integrate in the host genome. This property ensures replication of the therapeutic transgene during cell division and stable transmission to its progeny. Although a substantial part of gene therapy studies and clinical trials (64.5%) focuses on the treatment of cancer, here we will address only gene therapy for inherited monogenic diseases and its foreseen use in the Turkish population. Discussion of gene therapy for multifactorial diseases or malignancies is beyond the scope of this article and has been reviewed recently by Sanlioglu (2016).

Development of retroviral and lentiviral vectors for HSC gene therapy

The gammaretroviral genome (γRV) consists of a ssRNA and is approximately 7-10 kb in length. Retroviral vectors can stably integrate into the host genome using the enzymes reverse transcriptase (RT), which turns ssRNA into ssDNA and integrase (IN), which facilitates integration into the host genome. Gammaretroviral vector proviral DNA consists of a 5' and a 3' long terminal repeat (LTR), consisting of a U3, R and a U5 region, as well as open reading frames (ORF) for structural (Naldini *et al.* 1996), replication (pol) (Puthenveetil *et al.* 2004) and envelope proteins (Maetzig *et al.* 2011). The first retroviral constructs have been developed almost 40 years ago and were initially based on murine leukemia virus (MLV) (Shimotohno & Temin 1981, Wei *et al.* 1981).

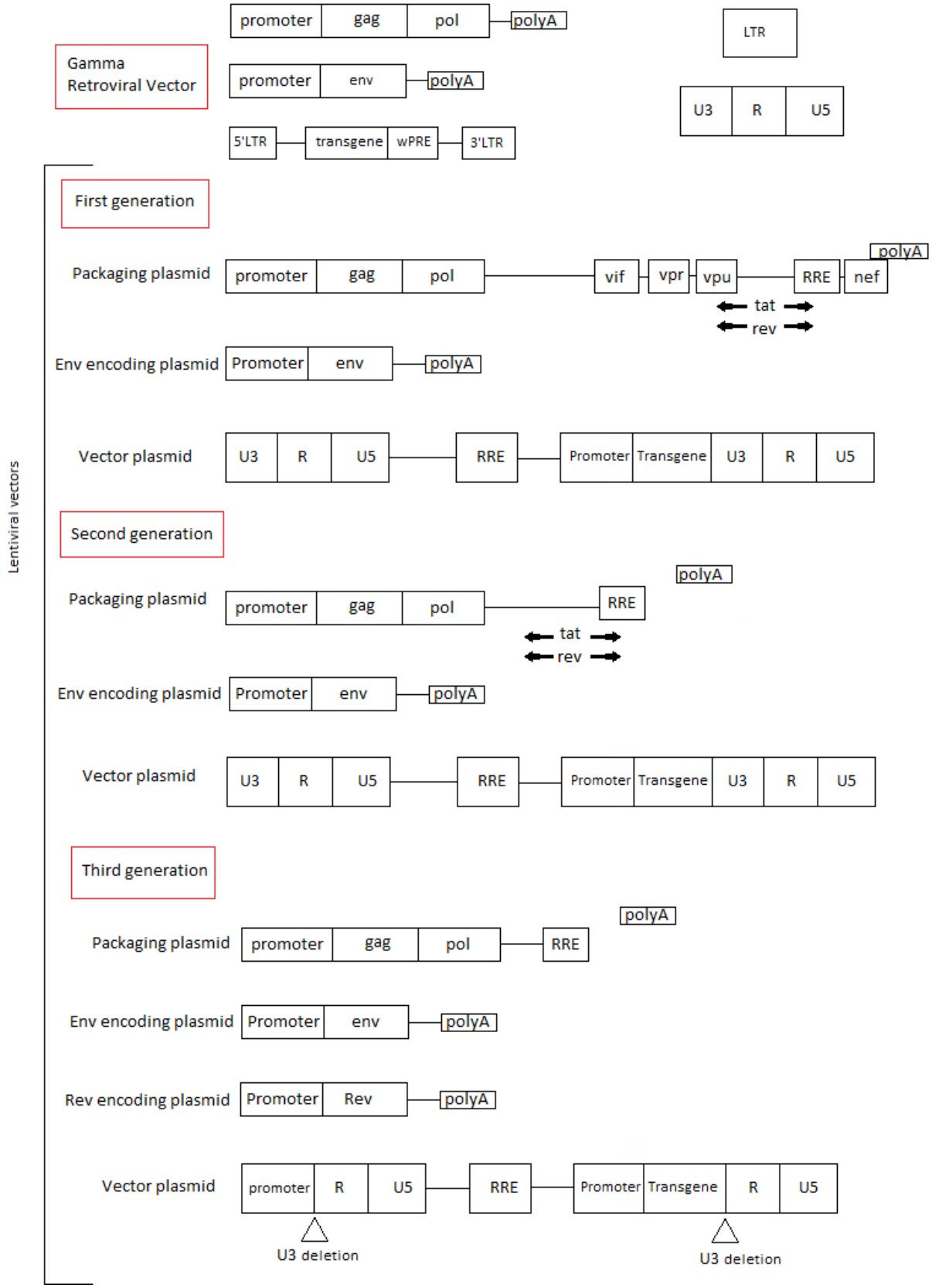

Fig. 1. Development of different generations of retroviral and lentiviral vectors. To generate second (ΔVpr, Vif, Vpu, Nef) and third (also ΔTat) generation vector systems, genes not required for transduction were deleted. To improve safety, the *rev* gene was deleted from the packaging construct and the promoter-enhancer sequences in the 5'LTR U3 region of the integrated transgene were deleted, creating self-inactivating (SIN) lentiviral vectors.

Table 1. Design and safety features of different retroviral and lentiviral constructs.

Vectors	Plasmids	Features	Insertional mutagenesis	Ref
γRV (MLV)	1) gag, pol, env; 2) vector genome and transgene	genome integration, restricted to dividing cells, RCR	+	(Maetzig *et al.* 2011)
First generation LV	1) gag, pol, rev, vpi, vpu, vpr, nef; 2) env; 3) vector genome and transgene	genome integration, dividing and non-dividing cells, RCL	+	(Naldini *et al.* 1996)
Second generation LV	1) gag, pol, rev, Δvpr; 2) env; 3) vector genome and transgene	genome integration, dividing and non-dividing cells, low RCL	+	(Zufferey *et al.* 1997)
Third generation SIN-LV	1) gag, pol; 2) env; 3) rev; 4) vector genome and transgene	self-inactivating, genome integration, dividing and non-dividing cells, very low RCL	+/-	(Dull *et al.* 1998)
Fourth generation SIN-LTR1, Lenti-X	1) gag; 2) Δvpr, pol; 3) tat, rev; 4) env; 5) vector genome and transgene	Genome integration, dividing and non-dividing cells, very low RCL	+/-	(Berkhout 2017, Vink *et al.* 2017)
NILV	1) gag, Δpol (deletion of integrase); 2) env; 3) vector genome and transgene	No genome integration, dividing and non-dividing cells	-	(Banasik & McCray 2010, Shaw & Cornetta 2014, Wanisch & Yanez-Munoz 2009)

Since retroviruses use receptor binding to transfer their genetic material into specific somatic cells, this mechanism can be modified and used to transfer transgenes for therapeutic intervention into specific target cells, eg. HSCs and treat monogenic diseases. To prevent the generation of replication competent retrovirus (RCR) in genetically modified cells, the genes for Gag/Pol and Env need to be separated using a split packaging design. The resulting retroviral vector contains the packaging signal (ψ), the primer binding site (PBS) and the long terminal repeats (LTR), but carries the transgene instead of the Gag/Pol and Env genes, which are expressed by separate plasmids. After integration of the retroviral DNA into the host cell chromosomal DNA, the proviral DNA is replicated during the cell cycle, and subsequently passed to the cell's progeny (Buchschacher & Wong-Staal 2000, Schambach *et al.* 2009, Sinn *et al.* 2005). However, γRV vectors can only infect dividing cells and permanent insertion has been shown to be associated with a risk for insertional mutagenesis (Hacein-Bey-Abina *et al.* 2003, Temin 1990).

In the 1990s, Naldini *et al.* (1996) developed lentiviral vectors based on the Human immunodeficiency virsus-1 (HIV-1). Lentivirus (LV) has the same basic biological features as γRV (Nisole & Saib 2004). However, LV vectors have the additional ability to transfer genetic material to both dividing and non-dividing cells and are considered safer than γRV (Lewis & Emerman 1994, Schambach *et al.* 2013). Lentiviruses, when compared to oncogenic γRV, have a more complex genome and have two regulatory genes, tat and rev, which are indispensible for viral replication and four unneccessary accessory genes, vif, vpr, vpu and nef, which are key for *in vivo* replication and pathogenesis of HIV-1 but not required for *in vitro* viral expansion or transduction.

The first generation LV vectors developed by Naldini *et al.* (1996) consisted of a three plasmid system. The first plasmid contained the genes for gag, pol, tat and rev, as well as the accessory genes and the packaging signal, the second plasmid encoded the heterologous env protein, which determines vector tropism and the third plasmid included the transgene or gene of interest (GOI) (Naldini *et al.* 1996). To generate second (ΔVpr, Vif, Vpu, Nef) (Dull *et al.* 1998) and third (also ΔTat) (Miyoshi *et al.* 1999) generation vector systems, genes not required for transduction were deleted and to increase safety, the *rev* gene was also deleted from the packaging construct, making expression of *gag* and *pol* strictly dependent on Rev complementation *in trans* on a separate plasmid (Dull *et al.* 1998). Therefore, in third generation LV vectors, generation of RCL requires two recombination events. Furthermore, as an additional safety feature, the promoter-enhancer sequences in the 5'LTR U3 region of the integrated transgene were deleted, creating self-inactivating (SIN) lentiviral vectors with a decreased risk for generation of replication competent lentivirus (RCL) (Zufferey *et al.* 1997). However, third generation LV vector genomic RNA still requires sequences to partially overlap wild-type HIV-1 gag and env genes for packaging into vector particles. To circumvent this problem and further decrease the total HIV-1 content in the LV vectors, a fourth generation LTR1 vector has been designed to prevent potential transfer of HIV-1 packaging sequences

to host cells by building a system wherein reverse-transcription results in single strand transfer, instead of the usual two (Berkhout 2017, Vink *et al.* 2017). Here, the lentiviral vector was further modified by placing several essential RNA signals (PBS-Ψ-RRE) outside the viral backbone and downstream of the 3'LTR (Berkhout 2017, Vink *et al.* 2017). Alternatively, in the Lenti-X packaging system the *gag* and *pol* genes are further separated onto two plasmids, requiring at least three low-frequency recombination events to generate RCL. Although retroviral vectors were shown to have a preference for integrations near specific proto-oncogenes (*LMO2, CCND2, MSD1-EVI1, PRDM16, SETBP1*) (Deichmann *et al.* 2007, Howe *et al.* 2008, Schwarzwaelder *et al.* 2007), SIN-LV vectors, which are considered less genotoxic than the γRV vectors, also carry non-negligible risks of insertional transformation (Modlich *et al.* 2009). Therefore, the last major advance has been the development of non-integrating LV (NILV) or integration deficient lentiviral (IDLV) vectors. This design aims to remove genome integration and eliminate any risk of recombination and insertional mutagenesis. NILV vectors can be made by inducing mutations in *pol* that alter the integrase protein and affect its function, or alternatively by deletion of a 12 bp fraction of the 5'LTR U3 unit and an 11 bp fraction of the 3'LTR U5 resulting in mutations in the integrase DNA attachment site (LTR *att* sites) (Banasik & McCray 2010). Despite these modifications, the vectors maintain the ability to enter target cells, perform reverse transcription, transport the pre-integration complex (Hacein-Bey Abina *et al.*) into the nucleus and efficiently express their transgene product. The modifications also do not affect the capacity of NILV vectors to efficiently infect both dividing and non-dividing cells (Shaw & Cornetta 2014, Wanisch & Yanez-Munoz 2009). The design and safety features of different RV and LV vectors are summarized in Table 1 and the most commonly used vector constructs are depicted in Fig. 1.

Status quo of ex vivo gene therapy for inherited monogenic diseases

Primary immune deficiencies

Primary immune deficiencies (PIDs) are the result of mutations in genes required during development of specific leukocytes, i.e. T, B and/or NK cells. Until recently, allogeneic hematopoietic stem cell transplantation (HSCT) was the only curative treatment option for PID patients. However, the outcome of HSCT in SCID depends on many factors including histocompatibility, conditioning regimen, manipulation of the graft, and T-cell depletion (Friedrich & Honig, 2010). In addition, allogeneic HSCT is often complicated by severe side effects resulting from both the conditioning regimen and graft versus host disease (GvHD). In cases where a suitable donor is unavailable, gene therapy offers the advantage of using the patient's own cells (autologous), thus preventing the immunologic complications related to GvHD. Moreover, the selective growth advantage of the genetically modified immune competent cells allows for minimal or no conditioning (EBMT/ESID guidelines for haematopoietic stem cell transplantation for primary immunodeficiencies 2017). Recent clinical trials using gene therapy have led to (partial) immune restoration in patients with X-linked SCID (X-SCID), adenosine deaminase (ADA)-deficient SCID, Wiskott-Aldrich syndrome (WAS) and chronic granulomatous disease (CGD).

The most common PID is X-linked SCID. The disease is the result of a mutation in the *IL2RG* gene encoding the common gamma chain (γc) cytokine receptor subunit. This subunit is shared by the receptors for IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21. Abnormal signaling through these receptors results in disruption of the development of T- and NK-cells and B-cells, although present, cannot function properly due to the absence of T-cells. Between 1999 and 2006, 20 children for whom an HLA-compatible donor was not available, were treated in two gene therapy trials for X-SCID in Hôpital Necker, Paris (Cavazzana-Calvo *et al.* 2000, Hacein-Bey-Abina *et al.* 2002) and Great Ormond Street Hospital (GOSH, London) (Gaspar *et al.* 2004). The vectors were based on the use of γRV constructs in which the therapeutic gene, *IL2R*γ, was placed under the transcriptional control of the LTR, with the use of either an amphotropic envelope (Cavazzana-Calvo *et al.* 2000) or the gibbon ape leukemia virus (GALV) envelope (Gaspar *et al.* 2004). All children received *ex vivo* transduced CD34+ cells without preconditioning. Doses ranged from 5 to $20x10^6$ CD34+ γc+ cells/kg weight. Between 31-68 months after infusion of the gene modified cells, 4 of the 10 patients from the Paris trial (Hacein-Bey-Abina *et al.* 2008) and 1 of the 10 patients in the London trial (Howe *et al.* 2008) developed a T-cell lymphoproliferative disorder. In the Paris trial, 2 patients had blast cells with activating vector insertions near the LIM domain–only 2 (*LMO2*) protooncogene (Hacein-Bey-Abina *et al.* 2003), 1 patient had blast cells with an integrated vector near *LMO2* and a second integrated vector near the proto-oncogene *BMI1* and in the following patient, blast cells contained an integrated vector near a third proto-oncogene, *CCND2* (Hacein-Bey-Abina *et al.* 2008). The patient in the London trial developed T-ALL, and integration of the vector was found in an antisense orientation 35 kb upstream of *LMO2* (Howe *et al.* 2008). Although the role of *LMO2* alone in oncogenesis has been shown in primary T-cell leukemia (Royer-Pokora *et al.* 1991) and hematopoietic tumors in mice (Dave *et al.* 2004), leukemogenesis in these patients was likely the result of overexpression of *LMO2*, followed by the occurrence of other genetic abnormalities unrelated to vector insertion, including a gain-of-function mutation in *NOTCH1*, deletion of the tumor suppressor gene locus cyclin-dependent kinase 2A (*CDKN2A*), and translocation of the TCR-β region to the *STIL-TAL1* locus (Hacein-Bey-Abina *et al.* 2008, Howe *et al.* 2008). Chemotherapy led to sustained remission in 4 of the 5 patients and was associated with restoration of polyclonal gene-corrected T-cell populations with a fully diverse T-cell repertoire.

The remaining subject passed away from refractory leukemia despite chemotherapy. As a result, a total of 19 out of 20 patients benefited from the therapeutic gene transfer (Hacein-Bey-Abina *et al.* 2008, Howe *et al.* 2008) and up to 10 years later, 17 were alive and maintained (nearly) full correction of the T-cell immunodeficiency (Cavazzana-Calvo *et al.* 2000, Hacein-Bey-Abina *et al.* 2002, Howe *et al.* 2008). Gene therapy at NIH in 3 children from 10 to 14 years of age, receiving up to $30x10^6$ CD34+ γc+ cells/kg was moderately successful (Chinen *et al.* 2007), whereas treatment of two older patients (20 years old) and receiving $0.8x10^6$ and $4.5x10^6$ CD34+ γc+ cells/kg, respectively, completely failed to produce lasting recovery of T-cell immunity (Thrasher *et al.* 2005). Increasing age, as well as a clinical history of chronic infection and GvHD negatively affect the chances of the gene transfer treatment to restore effective thymopoiesis. In addition, thymus hypoplasia as a result of prolonged absence of interaction between T-precursor cells and thymic epithelium can become irreversible. Therefore, it is strongly recommended that gene therapy in eligible SCID patients should be done as soon as possible after diagnosis (Aiuti & Roncarolo 2009).

Adenosine deaminase (ADA) is an essential enzyme of the purine metabolism, which catalyzes the deamination of adenosine and deoxyadenosine in the purine catabolic pathway after DNA breakdown and is expressed in all tissues of the body (Blackburn & Kellems 2005). ADA deficiency is a fatal, autosomal recessive disease and results in the intracellular accumulation of its metabolic substrates, dAXP and adenosine, causing toxicity not only in lymphoid progenitor cells, but also in bone, brain, lungs, liver and epithelia. The condition is particularly severe as the development of T, B and NK-cells is affected, resulting in defects in both cell-mediated and humoral immunity (Blackburn & Kellems 2005). The disease can be treated by HLA-identical HSCT (Antoine *et al.* 2003), with current survival rates up to 90% if treated at an early time point (Gaspar *et al.* 2009), but the use of alternative donors, such as matched unrelated donors (MUD) or umbilical cord blood (UCB), is associated with a high risk of (treatment-related) death or lack of engraftment (Gaspar 2010). Alternatively, enzyme replacement therapy (ERT) with Poly-ethylene glycol-modified ADA (Peg-ADA) can be started (Hershfield 1995). However, comparison of HSCT and ERT showed that where HSCT results in a constant peripheral T-cell number and diversification of the T-cell repertoire and an increase in B-cells, ERT results in a slowly progressive narrowing of the T-cell repertoire and a decrease in CD19+ lymphocytes in comparison to age-matched children (Serana *et al.* 2010). Initially, ADA-SCID patients were treated with retrovirally transduced peripheral blood lymphocytes (PBL) or autologous UCB (Kohn *et al.* 1998). Most patients displayed long-term persistence of transduced T-cells in the circulation, in the absence of toxicity, but due to low efficiency of gene transfer and engraftment, correction of the immunologic and metabolic defects was insufficient and all patients continued to receive ERT (Aiuti *et al.* 2002, Blaese *et al.* 1995, Bordignon *et al.* 1995, Kohn *et al.* 1998). Subsequently, more than 30 ADA-SCID patients have been treated with gene therapy in different transplantion centers worldwide (Cappelli & Aiuti 2010, Ferrua *et al.* 2010a). 15 patients were treated with retrovirally transduced autologous HSC in HSR-TIGET, Italy (Aiuti *et al.* 2009, Aiuti & Roncarolo, 2009, Aiuti *et al.* 2002), 10 in CHLA-NIH, USA (Engel *et al.* 2007, Sokolic *et al.* 2008), 5 in GOSH, UK (Gaspar *et al.* 2009, Gaspar *et al.* 2006), and 2 in Hokkaido, Japan (Otsu *et al.* 2006). The patients from the Italy trial displayed progressive immune reconstitution and long-term multilineage engraftment and were able to discontinue ERT treatment in 13 of the 15 cases (Aiuti & Roncarolo 2009). In contrast to the gene therapy trials for X-SCID, no cases of leukemia related to insertional mutagenesis have been reported in patients treated for ADA-SCID (Aiuti *et al.* 2007), despite the use of a similar γRV vector and the observation of a similar frequency of integration near *LMO2* and other proto-oncogenes (Aiuti *et al.* 2007). These differences have been attributed to the use of different internal promoters and the transgene itself. These positive results have resulted in the approvement of Strimvelis, the first gene therapy drug for treatment of children with ADA-SCID, in 2016 by the European Commission (Aiuti *et al.* 2017, European Medicines Agency 2016).

X-linked CGD is an inherited primary immunodeficiency disease, due to a defect in the gp91phox gene that encodes a NADPH oxidase transmembrane protein. CGD affects phagocytes resulting in defective intracellular killing (Kang *et al.* 2011), causing recurrent bacterial and fungal infections. The first gene therapy trials for CGD took place at the NIH in the 1990s using γRV vectors (Malech *et al.* 1997). However, these gene therapy trials failed to sustain long-term engraftment of the retrovirally transduced CD34+ cells (Grez *et al.* 2011). In order to improve engraftment of transduced CD34+ cells, low dose non-myeloablative conditioning was used in following studies in two adults and two children. This resulted in improved engraftment and temporary clinical benefit. However, due to insertional activation of the proto-oncogene *MDS1-EVI1* and transgene silencing, this clinical benefit was eventually lost and all patients developed myelodysplastic syndrome (MDS) (Ott *et al.* 2006, Siler *et al.* 2015, Stein *et al.* 2010). In total, 5 clinical trials were performed using γRV vectors and cell engraftment progressively decreased with time in all 12 patients, with several patients developing MDS. In contrast to the gene therapy of other immune deficiencies, corrected HSCs did not appear to have a selective growth advantage. Furthermore, it has been suggested that the constitutive expression of gp91phox in HSCs may have led to overproduction of ROS, causing cellular toxicity and loss of the genetically corrected stem cells over time (Arnold & Heimall 2017). Current gene therapy trials for CGD make use of codon-optimized third generation SIN-LV vectors to decrease the risk for insertional mutagenesis (Keller *et al.* 2018),

and specific internal promoters designed to allow transgene expression restricted to the myeloid lineage only using a chimeric promoter (Santilli *et al.* 2011) or a myeloid specific promoter (Chiriaco *et al.* 2014).

Wiskott-Aldrich Syndrome (WAS) is a disorder caused by a mutation in the *WAS* gene that encodes WASp, a regulator protein of the main actin cytoskeleton and resulting in eczema, microtrombopenia, infections and autoimmunity in patients. In the first clinical trial for WAS a γRV vector was used, which resulted in correction of the functional defect. However, 7 of the 9 treated patients developed leukemia, because of integrations in the *LMO2*, *MDS1/EVI1*, *MN1* proto-oncogenes (Ghosh & Gaspar 2017). The use of a SIN-LV-WASp vector to treat an adult WAS patient and 10 children showed promising results with rapid engraftment and sustained clinical improvement in the absence of insertional mutagenesis, showing that LV-based gene therapy may be developed as an alternative treatment for WAS (Hacein-Bey Abina *et al.* 2015, Morris *et al.* 2017).

Hemoglobinopathies

β-Thalassemia (β-Thal) and Sickle Cell Disease (SCD) are the most common inherited monogenic disorders throughout the world, with more than 300,000 affected neonates born annually. Hemoglobinopathies are particularly common in Asian and Mediterranean countries, one of which is Turkey (Higgs *et al.* 2012). Especially, the incidence of β-Thal in the Marmara region (up to 11.7%) and southern parts (up to 13.1%), and SCD (up to 47%) in Çukurova region of Turkey are very high (Kilinc 2006). According to a survey conducted by the Ministry of Health and hemoglobinopathy council in 2006, 5000 β-Thal cases have been reported in Turkey, whereas the prevalence of SCD in Turkey is estimated to be 0.03-0.06%. Both β-Thal and SCD are recessively inherited hemoglinopathies characterized by a mutation in the hemoglobin β (HBB) gene. In SCD, a point mutation results in the formation of hemoglobin S (HbS), which polymerizes in the deoxygenated state, resulting in red blood cell sickling. Most patients suffer from chronic hemolytic anemia, acute and chronic pain, pulmonary and renal failure, cardiovascular disease and a cognitive decline. β-Thal results in reduced or abrogated β-hemoglobin production and ineffective erythropoiesis. Thalassemia major patients are transfusion dependent and suffer from severe hemolytic anemia, iron overload, hepatosplenomegaly, cardiomyopathy, endocrine disorders and skeletal abnormalities due to bone marrow expansion. The only current treatment of hemoglinopathies is allogenic HSCT from an HLA-matched donor. Genetic correction of autologous HSCs could serve as an alternative treatment option for hemoglinopathies in the absence of a suitable donor.

In order to increase the safety profile of LV vectors and improve expression of the Hb transgene, Puthenveetil *et al.* (2004) designed a 1.2 kb cHS4 insulator sequence, which blocks enhancer activity, preventing activation of nearby oncogenes, and reduces silencing of the transgene by heterochromatin. Further modifications of this insulator were used in following trials. In 2007, a single patient was infused with autologous CD34+ HSCs transduced with the HPV569 SIN-LV vector containing two copies of the cHS4 chromatin insulator in the U3 region and encoding a mutated adult β-globin ($\beta^{A(T87Q)}$) with anti-sickling properties (Cavazzana-Calvo *et al.* 2010, Negre *et al.* 2015,). Three years after transplantation, the patient was tranfusion independent with a stable level of 8-9 g/dL Hb. A subsequent trial used an optimized version of the LV construct (Negre *et al.* 2015), without the cHS4 insulator and included a total of 18 patients with β-Thal and 4 patients with SCD BB305. All patients (n=22) showed a highly polyclonal integration profile, with no clonal dominance, had stable Hb levels and became transfusion-indepent within 12 months after GT (n=15) or required significantly less red blood cell transfusions (n=7) (Cavazzana 2016, Thompson *et al.* 2016). The gene therapy trial resulted in stable hematopoetic reconstitution, was well tolerated and did not cause severe adverse events. A clinical trial in 2012 in four patients using the wild-type β-globin transgene resulted in limited gene transfer and lack of efficacy (Mansilla-Soto *et al.* 2016). In 2015, a clinical trial phase I/II was started in Italy for transfusion dependent patients. HSCs were transduced with the GLOBE SIN-LV vector, which expresses the wild-type β-globin transgene under the control of the β-globin promoter (Miccio *et al.* 2008, Roselli *et al.* 2010). In 2017, seven patients (3 adults and 4 children) with different genotypes had been enrolled and were treated with GLOBE-SIN-LV transduced $CD34^+$ cells at a dose of $16x10^6$-$19.5x10^6$ cells/kg (Marktel *et al.* 2017). The median follow-up was 13 months. The procedure was generally well tolerated by all patients, with no product-related adverse events, no evidence of RCL or abnormal clonal proliferation (Marktel *et al.* 2017). Patients showed polyclonal multilineage engraftment, and three of the four treated children became transfusion independent, whereas transfusion requirements decreased in adults, indicating that age of treatment may be an important factor determining the efficacy of the procedure.

Preclinical studies for gene therapy of SCD demonstrated that gene therapy improved sickling. One patient in France was treated with BB305 lentiviral vector transduced HSCs. The patient was followed during two years and became transfusion independent with stable Hb levels of 12 g/dL with therapeutic Hb and HbS accounting for 48% and 46% of the Hb tetramers, respectively (Ribeil *et al.* 2017). In an extended clinical phase I/II trial using this vector, peripheral blood levels of the BB305 vector remained low in all treated SCD subjects, with no evidence of clinical benefit (Kanter *et al.* 2016). In another two gene therapy clinical trials, an anti-sickling γ/β-globin transgene (sGbG) (Perumbeti *et al.* 2009) and a β-globin transgene with three anti-sickling point mutations (Lenti-bAS3-FB) (Romero *et al.* 2013) are being assessed currently.

Metabolic Disorders

Adrenoleukodystrophy (ALD) is an X-linked disease caused by a defect in the ABCD1 (ATP-binding cassette subfamily D) gene which encodes the transporter of the ALD protein (ALDP). ALD was first described in 1992. ALD triggers accumulation of fatty acids that damage the myelin sheaths of neurons, causing motor and cognitive impairment. Allogeneic HSCT is the treatment of choice if a suitable donor is available (Shapiro *et al.* 2000). Lentiviral gene therapy is currently being developed based on promising results showing ALDP expressing human microglia in the brains of NOD/SCID mice after xenotransplantation of lentivirally transduced ALDP expressing human CD34+ HSCs (Benhamida *et al.* 2003). Preliminary results from HSC gene therapy indicated that a limited number of microglia cells may need to be corrected to prevent the demyelinating process (Cartier & Aubourg 2010, Cartier *et al.* 2009). Autologous CD34+ cells genetically corrected using a lentiviral vector encoding the wild-type ABCD1 gene were reinfused into two boys with progressive cerebral demyelination. The boys displayed polyclonal, multilineage engraftment and no evidence of clonal dominance. Cerebral demyelination was arrested at 14 and 16 months, respectively, without further progression (Cartier *et al.* 2012, Cartier *et al.* 2009). In a subsequent phase II/III safety and efficacy clinical study, 17 boys with cerebral X-ALD with early-stage disease and gadolinium enhancement on magnetic resonance imaging (MRI) were infused with autologous CD34+ cells transduced with the elivaldogene tavalentivec (Lenti-D) lentiviral vector. After 29 months of follow-up, all patients had multilineage engraftment of genetically modified cells, with no evidence of preferential integration near known oncogenes or clonal expansion. Although 2 of these 17 patients showed disease progression, the remaining 15 patients had stable expression of the ALD protein (Eichler *et al.* 2017).

Metachromic Leukodystrophy (MLD) is a neurodegenerative lysosomal storage disorder caused by arylsulfatase A (ARSA) deficiency and is autosomal recessively inherited. ARSA deficiency causes accumulation of sulphatide in the CNS, kidney, peripheral nerves, pancreas and liver. The disease affects children and results in premature deaths. Due to their cerebral involvement, both HSCT and development of ERT have been shown to be of limited success (Biffi *et al.* 2011, Boelens, 2006, Boelens *et al.* 2010, Rovelli & Steward 2005). In previous studies, expression of ARSA was detected after transplantation of LV corrected HSCs in a MLD mouse model (Biffi *et al.* 2004, Biffi *et al.* 2006). Recently, a phase I/II clinical trial of MLD was performed in Italy. Three children with ARSA deficiency and mutations associated with early-onset MLD were treated at the presymptomatic stage and received autologous HSCs transduced with a lentivirus carrying the *ARSA* gene. All three patients showed high-level polyclonal, multilineage engraftment of the transduced HSCs and high ARSA activity was detected in the hematopoietic lineages and in the cerebrospinal fluid resulting in arrested progression of neurodegenerative symptoms (Biffi *et al.* 2013). No evidence of clonal expansion was detected, indicating that gene therapy may be a good alternative to HSCT, especially since the effects of ERT for storage diseases with cerebral brain involvement are limited.

For diseases such as Gaucher, Fabry and Pompe disease, ERT is available and effective. However, the development of gene therapy may still serve a purpose because of the impact on the patient (requiring recurrent visits to the hospital for ERT infusion), the cost-effectiveness of the procedure (ERT is relatively expensive in comparison to a single gene therapy treatment) and because gene therapy of autologous HSCs is in intention a single, curative treatment and minimally invasive, increasing the quality of life of patients.

For other metabolic diseases for which allogeneic HSCT is possible, in the absence of a matched donor, gene therapy offers the advantages of decreasing morbidity related with allogeneic HSCT such as myeloablative conditioning and GvHD. Ongoing developments have made the used lentiviral vectors increasingly safe and promising results are obtained in clinical trials in most of the inherited monogenic diseases. An overview of the results of gene therapy trials for diseases discussed above is given in Table 2.

New developments for improved lentiviral vector biosafety and efficacy

Gamma retroviral vectors have been shown to preferentially integrate near transcriptional start sites and regulatory gene regions of specific proto-oncogenes, such as *LMO-2*, *CCND2*, *MDS1/EVI1*, *PRDM16*, *SETBP1*, *MECOM* (Howe *et al.* 2008) and many of the initial clinical trials for X-linked SCID, CGD and WAS have shown the risks related to the use of γRV (Ghosh & Gaspar 2017, Hacein-Bey-Abina *et al.* 2008, Howe *et al.* 2008, Ott *et al.* 2006). To reduce the risks of insertional mutagenesis, third generation self-inactivating retroviral and lentiviral vectors were developed (Maetzig *et al.* 2011), which have been shown to have a signficantly improved safety profile (Bordignon *et al.* 1995, Cartier *et al.* 2012, Cartier *et al.* 2009, Cavazzana 2016, Hacein-Bey Abina *et al.* 2015, Kohn *et al.* 1998, Morris *et al.* 2017, Thompson *et al.* 2016).

Although the SIN-LV vectors, which display a preferred integration in transcribed genes, are considered less genotoxic than the SIN-γRV vectors, the risks of insertional transformation of HSCs by both SIN-LV and SIN-γRV vectors remains present and appears to be dictated largely by the type of internal promoter used and the transgene itself (Modlich *et al.* 2009). Physiological (internal) promoters are generally weaker insertional mutagens in comparison to retroviral promoters (Zychlinski *et al.* 2008) and using lineage or tissue-specific promoters can therefore potentially increase biosafety (Pauwels *et al.* 2009). Furthermore,

the use of tissue-specific promoters or inducible promoters over constitutively active promoters may result in a more physiological expression pattern of the transgene, which may be preferred to avoid cellular toxicity related to overexpression of transgenic proteins (Arnold & Heimall 2017), as well as to avoid an immunological response against the transgenic protein. *In vitro* quantitative assays, such as immortalization tests, demonstrated that the risk of insertional activation of proto-oncogenes is directly related to the strength of the enhancer sequences contained in the vector (Schambach *et al.* 2013). One way to overcome this problem is to introduce insulators, such as cHS4, which can establish bounderies between regulatory sequences, into the viral genome (Gaszner & Felsenfeld 2006, Puthenveetil *et al.* 2004), both preventing activation of nearby proto-oncogenes and at the same time protecting the transgene from silencing by spreading heterochromatin. The design of novel pseudotypes may further increase cell-specific targeting during transduction of mixed populations of unstimulated hematopoietic stem and progenitor cells, which will improve transduction efficiency (Frecha *et al.* 2008). In addition, current developments with regards to direct targeting of viral integration into "safe sites" of the genome (gene addition) or targeted integration into the site of the mutated gene (gene replacement) using zinc finger nucleases (Porteus & Carroll 2005) or CRISPR-Cas9 nucleases will greatly improve physiological regulation of the transgenic gene and biosafety.

Table 2. Results from clinical trials of hematopoietic stem cell gene therapy for inherited monogenic diseases.

Disease	Phenotype	Affected gene	Vector	Current Rx	Side effects	Clinical benefit	References
X-SCID	Absence of T and NK cells and mature B cells	IL2Rγ	γRV	HSCTx	Insertional mutagenesis	Selective growth advantage of transduced cells Immunological reconstitution	(Cavazzana-Calvo *et al.* 2000, Hacein-Bey-Abina *et al.* 2008, Hacein-Bey-Abina *et al.* 2002, Howe *et al.* 2008)
ADA-SCID	Immunodeficiency neurological abnormalities	ADA	γRV	ERT HSCTx	-	Selective growth advantage of transduced cells, immunological reconstitution, clinical improvement, discontinuation of ERT treatment	(Aiuti *et al.* 2007, Aiuti & Roncarolo 2009, Cappelli & Aiuti 2010, Ferrua *et al.* 2010b)
X-CGD	Non-functional phagocytes	Gp91phox	SIN-LV	HSCTx	Insertional mutagenesis	No selective growth advantage of transduced cells Cellular toxicity, loss of corrected HSCs	(Keller *et al.* 2018, Stein *et al.* 2010)
WAS	Microthrombocytopenia, autoimmunity	WASP	γRV	HSCTx	Insertional mutagenesis	-	(Ghosh & Gaspar 2017)
			SIN-LV		-	Multilineage engraftment, clinical improvement, no clonal expansion	(Hacein-Bey Abina *et al.* 2015, Morris *et al.* 2017)
β-Thal	Hemolytic Anemia	β-globin	SIN-LV	HSCTx	-	Polyclonal integration, no clonal dominance, multilineage engraftment, transfusion indepence	(Cavazzana-Calvo *et al.* 2010, Cavazzana 2016, Thompson *et al.* 2016)
SCD	Hemolytic anemia	β-globin	SIN-LV	HSCTx	-	Minimal clinical benefit	(Marktel *et al.* 2017)
X-ALD	Cerebral demyelination	ABCD1	SIN-LV	-	-	Arrested progression of demyelination, polyclonal, multilineage engraftment and no clonal dominance	(Cartier *et al.* 2012, Cartier *et al.* 2009, Eichler *et al.* 2017, Kohn *et al.* 1998)
MLD	Cerebral demyelination	ARSA	SIN-LV	HSCTx	-	High-level polyclonal, multilineage engraftment, arrested progression of neurodegenerative symptoms, no clonal expansion	(Biffi *et al.* 2013, Bordignon *et al.* 1995)

Current status of gene therapy research in Turkey

Very few research groups in Turkey are currently working on the development of gene therapeutical approaches for inherited diseases. According to the website of the Scientific and Technological Research Council of Turkey (TÜBİTAK, https://www.tubitak.gov.tr) genetic modification related R&D activities were concentrated in projects in the Genetic Engineering and Biotechnologies Institute of MAM (Marmara Research Center). These projects however, were more focused on the development of different transgenic mouse models, development of vaccines or development of non-viral vectors. Search queries for research projects using "lentiviral" and "vector" as search items using the TÜBİTAK database for supported projects (www.cabim.ulakbilim.gov.tr/tr-dizin/tubitak-destekli-projeler-veritabanı) resulted in a total of 60 hits, of which only 1 focused on gene therapy. However, since this database has not been updated since 2013, these numbers are most likely not representative for the actual numbers of currently ongoing gene therapy related projects.

Why is the development of gene therapy important for Turkey?

Consanginous marriages increase the risk of autosomally inherited diseases. The highest rates of consanginous marriages occur in North and sub-Saharan Africa, the Middle East, and West, Central and South Asia. According to the Turkish statistical institute stastistic (TÜİK), the rate of consanginous marriage rates in Turkey is approximately 21%. Of these, 35% are located in rural areas and 20-25% are located in urban areas. Within Turkey, the rates of consanginous marriages are highest in Southeastern Anatolia (40.4%) and lowest in Western Marmara (4.8%). Overall in Turkey, one in five marriages is between closely related family members, of which 70% between first cousins. Marriages between first and second cousins increases the risk of post-natal infant mortality significantly, with 45.9/1000 infant deaths in unrelated marriages, versus 72.1 infant deaths in consanguineous marriages (Sağlık Bakanlığı, 2002). This high level of consanguinity in Turkey results in a relatively high prevalence of PIDs and metabolic diseases in comparison to Europe and the USA and anually 4000 patients with possible PIDs are reffered to the 10 pediatric immunology centers in Turkey. In addition, according to an official publication by the Ministry of Health in 1996, the number of patients with diseases of blood or blood-forming organs, excluding anemias, reached 9597 within the years 1964-1994. Thus, the social economic burden of the not so rare diseases in Turkey is quite substantial.

For some of the inherited monogenic diseases allogeneic HSCT can be curative, and where outside of Turkey the search for an available, suitable donor may be difficult; in Turkey often matching donors can be found within families (Balci *et al.* 2011). However, even if available, both HSCT and ERT-related morbidity and frequent hospital visits, make the development of a single, curative treatment a tempting alternative. In addition, the costs related to HSCT, including conditioning and follow-up, and life-long treatment with ERT are considerable: In the USA, costs related to life-long ERT treatment for Fabry disease (Fabrazyme) are estimated to be $200,000/year per patient; Hurler Syndrome (Aldurazyme) $200,000/year per patient; Gaucher type 3 (Cerezyme) $200.000/year per patient; Pompe disease (Myozyme) $300.000/year per patient; Hunter Syndrome (Elaprase) $375.000/year per patient. Whereas the national health insurance systems in West Europe fully reimburse costs related to HSCT or ERT, other countries may not or not completely cover costs related to orphan drugs (Shah 2006), making the development of a single, curative treatment, such as gene therapy for these countries a high priority.

Recommendations for Turkey

The rationale of gene therapy is to repair, inactivate or to replace dysfunctional genes that cause disease with the aim of establishing or acquiring normal function. Hematopoietic stem cell gene therapy offers a possible curative treatment option for a range of patients with inherited monogenic diseases, who currently have no alternative treatment option, such as HSCT or ERT. Many gene therapy clinical trials have been performed mostly in Europe and the USA during the last twenty years for a wide range of immune deficiencies and metabolic diseases, of which only a fraction is discussed above. Although several Turkish patients have been treated with gene therapy for ADA-SCID, CGD and WAS, and patients enrolled in gene therapy clinical trials include a disproportionate number of children from Turkish origin, development of gene therapy or participation in gene therapy clinical trials for inherited monogenic diseases in Turkey is not currently actively pursued.

Gene therapy not only offers a curative treatment option, but also, as observed with the latest SIN-LV contructs, provides in general a good quality of life, low treatment-related morbidity, a decreased duration and frequency of hospitalization in addition to an improved clinical condition. However, the efficacy of the treatment is not only related to the number of cells infused, the vector copy number per cell or the level of transgene expression, but even more to the general condition of the patient upon start of the treatment. When gene therapy is done in eligible patients as soon as possible after diagnosis and preferably before irreversible symptoms occur, these patients have the best chances of good recovery and clinical improvement (Aiuti & Roncarolo 2009, Bordignon *et al.* 1995, Marktel *et al.* 2017, Thrasher *et al.* 2005).

Although current lentiviral vectors still harbor the intrinsic risk of insertional mutagenesis, recent clinical trials have shown that the actual occurrence of myelodysplastic syndromes and leukemogenesis is very low and that the benefits of the treatment outweigh the risks. Obviously, the long-term risks of the procedures cannot be fully appreciated, since gene therapy is just

coming of age, and assessment of these risks will require carefully planned, long-term follow-up of all treated patients. However, future improvements resulting in tissue-specific expression of genes, replacement of genes rather than addition, and increased biosafety will make hematopoietic stem cell gene therapy a powerful new tool to cure previously incurable patients. Therefore, the establishment of specialized centers in Turkey for gene therapy research, for the development of clinical gene therapy or participation in phase I/II gene therapy studies and for the production and quality control of gene therapy products should be stimulated.

In conclusion, lentiviral treatment of rare, inherited diseases is being rapidly developed and optimized and currently tested in multicenter preclinical trials. Although past experiences with retroviral and lentiviral gene therapy have shown that results should be assessed carefully, and long-term follow-up remains required, current data are very promising. Therefore, we recommend development of a specialized research center infrastructure that would allow participation of Turkey not only by providing patients or patient samples, but also by taking part at the research level and in the preclinical trials.

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Review

CANCER STEM CELL BIOLOGY

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Abstract: Cancer is becoming the leading cause of death all around the world. To develop better therapeutic options against cancer, we need a thorough understanding of tumor development and dissemination. As our knowledge increases, it becomes apparent that cancer is a very complex disease and this complexity is partially due to the great level of heterogeneity even within the same tumor mass. Therefore, there is a pressing need to decipher complex regulations and interactions of the tumor cells that lead to different hierarchies. Concepts of tumor-initiating and self-renewing stem cells have long been proposed to explain the emergence of a vast number of progenies within monoclonal neoplastic growth. It is now known that cancer stem cells which are found in many cancers have many roles in tumor development and dissemination. Many fascinating properties of cancer stem cells draw further attention to clarify their involvement in tumor cell plasticity, epithelial to mesenchymal transition, chemotherapy resistance and to develop therapeutic strategies for their targeting. Here we summarized recent efforts to illustrate the progress in our understanding of the biology of cancer stem cells.

Key words: Cancer stem cells, differentiation, plasticity, clonal selection, lineage tracing, and cancer therapy.

Özet: Kanser tüm dünyadaki ölümlerin en önde gelen sebebi olma yolunda ilerlemektedir. Kansere karşı daha etkili tedavi seçenekleri geliştirmek için tümör gelişimi ve yayılmasının çok daha iyi anlaşılması gerekmektedir. Konuyla ilgili bilgilerimiz arttıkça kanserin çok karmaşık bir hastalık olduğu ve bu karmaşıklığın kısmen aynı tümör kitlesinde dahi görülebilen yüksek heterojenlik düzeyine bağlı olduğu ortaya çıkmaktadır. Bu nedenle, farklı hiyerarşilere yol açan tümör hücreleri arasındaki karmaşık düzenleme ve etkileşimlerin açıklığa kavuşturulması için artan bir ihtiyaç söz konusudur. Tümör başlatan ve kendini yenileyen kök hücre kavramları, monoklonal tümör gelişiminde görülen çok sayıdaki neslin ortaya çıkışını açıklamak için uzun zamandan beri ileri sürülmektedirler. Artık günümüzde çoğu kanser tipinde bulunan kanser kök hücrelerinin tümör gelişimi ve yayılmasında çok sayıda rolleri olduğu bilinmektedir. Kanser kök hücrelerinin sahip oldukları pek çok ilginç özellikleri, tümör hücresi plastisitesi, epitemezenkimal dönüşüm ve kemoterapi direncindeki rollerinin açıklığa kavuşturulması ve yeni tedavi stratejilerinin geliştirilmesi için daha da ilgi çekmektedir. Bu çalışmada kanser kök hücresi biyolojisi ile ilgili bildiklerimizde yaşanan gelişmeleri ortaya koyabilmek için yapılan son çalışmalar özetlenmiştir.

Basic definitions: tissue stem cells vs. cancer stem cells

Stem cells (SCs) are described as having unlimited growth and division potential. They are hence called self-renewing cells of different adult tissues (Merrell & Stanger 2016). Although their regenerative potential is not as high as in the case of embryonic SCs (ESCs), tissue SCs can still differentiate into various cell types within a given tissue and therefore considered as being pluripotent (Grompe 2012). SCs are less differentiated relative to their increasingly better differentiated descendants. In contrast to SCs, normal somatic cells usually get arrested in a post-mitotic state where they will never return back to cell division cycle (Merrell & Stanger 2016). In normal tissue hierarchy, SC divisions usually give rise to two daughter cells, one of which remains to be a SC while the other is destined to be differentiated. This type of division is called asymmetrical since it does not yield identical daughter cells in contrast to symmetrical divisions (Morrison & Kimble 2006). The daughter cell which exits SC state is called progenitor cell or transit-amplifying cell since it still has the ability to continue cell division for a limited period of time. Many tissues, such as stomach, intestine and hair follicles, have examples of such intermediate cells before the emergence of fully differentiated descendants of the SCs (Rangel-Huerta & Maldonado 2017). Since the progenitors/transit-amplifying cells can continue to divide and form differentiated cells in the tissue, SCs do not need to divide continuously but periodically to generate progenitor cells which are responsible for most of the cell divisions occurring in the tissue (Rangel-Huerta & Maldonado 2017). By doing so, SCs are protected from mutations and other stressful insults associated with highly proliferative state.

Previously, SCs were thought to be mostly composed of quiescent SC niches which may be a significant fraction of the cells in different tissues (Clayton *et al.* 2007).

However, recent studies have put forward that in some epithelial tissues, like in intestine and stomach, SCs may be actively dividing throughout the life of the organisms (Barker *et al.* 2007, Barker *et al*. 2010). SCs also do not necessarily show asymmetric mitotic processing all the time (Batlle & Clevers 2017). In contrast to the classical view of asymmetric division, SC division may result in cells with different fates due to a process called neutral competition (Leushacke *et al.* 2013, Snippert *et al.* 2010, Doupe *et al.* 2010). The neutral competition occurs due to availability of different niches in the given tissue and may result in all SCs, all differentiated cells or mixed cell populations. The process of neutral competition is highly variable in outcomes yet it depicts great plasticity of SC niches in generating tissues like epidermis, intestine and stomach (Batlle & Clevers 2017). Overall, all SCs and the resulting daughter cells show great levels of plasticity in which even fully differentiated cells can re-gain stemness (hence called quiescent stem cells) or they may substitute upon loss of stem niche in various tissues (Li & Bhatia 2011). In some extreme cases with highly regenerative tissues like in liver, fully-differentiated hepatocytes can restore the lost tissue without reverting back to SCs (Stanger 2015). In most tissues, with rare exceptions like in hair follicle cells and nerve cells, it is not possible to find a quiescent SC (Hsu *et al.* 2014, Morizur *et al.* 2018). Therefore, it seems that nature has evolved diverse ways for tissue maintenance and repair in tissues of different origins even within the same organism.

Current efforts in cancer research have focused on understanding if mechanisms responsible for the tissue organization are functioning similarly in tumors as well. Thus, a similar model has been adopted by researchers trying to identify the interrelations of the cells within a tumor (Weinberg 2013). In this model, the tumor-initiating cells, which have the limitless self-renewal ability to generate vast number of neoplastic progenies are termed as cancer stem cells (CSCs). As we will explore in the following parts in more detail, CSCs are the only cell population in a given tumor which has the ability to initiate the formation of a whole new tumor when transplanted elsewhere or in a new organism (Nassar & Blanpain 2016). Indeed, surface marker sorted cell transplantation experiments resulted in identification of CSC populations in various cancers (Doulatov *et al.* 2012, Clevers 2011). Although CSCs and their progeny are genetically identical as in the case of asymmetric cell division in normal tissues, CSCs are the only cells with tumor-initiating and self-renewal potentials. The progeny cells, on the other hand, have lost their tumor-initiating abilities most probably due to being devoid of self-renewing capacity yet they may still be observed as highly proliferative (analogous to transit amplifying cells) during tumor progression. As our understanding on the cellular mechanisms regulating CSCs increases, it turns out to be more ostensible that these mechanisms are very similar to the ones that operate in normal tissue stem cells (Weinberg 2013). Hence, tumors do not develop new SC programs altogether. Instead they apply the basic rules operating in tissue SCs to adapt to the ever-changing conditions within the tumor microenvironment (Göktuna *et al.* 2018).

Identifying CSCs from tumors: transplantation vs. lineage-tracing

Xenotransplantation Experiments

Tumor progression usually occurs in a multistep manner in which tumor cells progressively acquire mutations leading to novel characteristics so that they can advance from benign to malign during the process. Moreover, not only different tumors from the same patient differ in composition of the cells but also high level of heterogeneity is observed even within the same tumor (Göktuna *et al.* 2018). All these different cells with different evolutionary history may lead to different cell populations harboring distinct mutations and characteristics (Magee *et al.* 2012). While some cells are highly proliferating, others can be observed to be senescent or dormant, yet others may be invasive and still others can be apoptotic all within the same tumor mass (Marusyk 2012). Although tumors arise from gradually altered normal tissue cells, many previous efforts to understand the origins of tumor cells have put forward that tumors are usually monoclonal (Novelli *et al.* 2003). In other words, tumors usually originate from a single ancestor cell which crosses over the border between normal and abnormal growth. The differences among tumor cells happen later on during the tumor development due to the highly mutagenic state of the tumor cells arising from fast proliferation (Nassar & Blanpain 2016). Especially, a recent report showed that intratumoral diversification occurs at the cellular level in colorectal tumors which have vastly increased their ability to mutate in a series of clonal expansion (Roerink *et al.* 2018). Clonal succession model is usually ascribed to explanation for this phenomenon. The model states that a mutant tumor cell, originated from the monoclonal tumor mass, may generate a vast number of progenies, and among these progenies, subsequent mutational events will cause additional floods of clonal expansion to generate new tumor subclones (Weinberg 2013). Therefore, the differences that lead to heterogeneity are gained later on during tumor progression.

Surprisingly, further experiments have proven that the cells resulting from a single clone were not always displaying the same characteristics and also not all of them were capable of generating new progenies within the neoplastic growth. In these experiments, human tumor cells were separated into distinct subpopulations via FACS which uses differential expression of various cell surface markers in diverse cell populations (Uçkun *et al.* 1995). The first experiments were carried out in hematological malignancies due to inherent qualities like less intratumoral heterogeneity and extensive knowledge of hematological cell surface markers. The first cancer where cell sorting utilized was acute myelogenous leukemia (AML) in which cells were sorted according to more or less differentiated phenotypes (Lapidot *et al.*

1994). Majority of the sorted cells were granulocytes and monocytes and these cells had very limited ability to proliferate in vivo while less well differentiated $CD34^{+}CD38^{-}$ cells (less than 1%) were actively proliferating and were able to form tumors when injected into immuno-compromised nude mice. Hence, only a small fraction of the tumor cells in AML was composed of tumorigenic and self-renewing cells which were later described as CSCs (Lapidot *et al.* 1994, Bonnet & Dick 1997). For a long time, similar observations in other hematological malignancies solidified CSC hypothesis. However, in solid tumors, CSCs could not be proven until successful sorting of breast cancer tumors yielded that about 2% of the cells were CSC ($CD44^{+}CD24^{-/low}$) and only 200 of such cells (as opposed to 10.000 cells from the original tumor mass) were sufficient to form tumors in xenotransplant experiments (Al-Hajj *et al.* 2003). This time, on the contrary, majority vs. minority of the sorted cell populations were showing comparable activity in their cellular growth while they were observed to display great differences in their tumorigenic abilities. Later on, self-renewing CSC subpopulations were demonstrated to be present in many other solid malignancies like hepatocellular, brain, lung, pancreatic, colorectal, head and neck cancers (Enderling *et al.* 2016, Nassar & Blanpain 2016). Therefore, results of all these experiments propose that organization of tumor cells shows similarities to normal tissues where minor groups of self-renewing stem cells can generate large number of cells while descendant cells have limited ability to do so. Currently, we also understand that these minority cell populations within tumors express distinct cell surface markers than that of non-SCs which further illustrates that these two cell populations most probably reside at different levels of differentiation.

Accumulating evidence from previous reports suggest that not in all cancers development of tumors were adhering to CSC model. As some critics of xenotransplantation assays reported that mouse leukemia cells, instead of human AML, were transplanted into immunocompetent mice and the CSC frequency was observed to be very high at about 10% or more (Kelly *et al.* 2007, Williams *et al.* 2007). Still other contrasting claims have resulted from serial transplantation experiments in mice. For instance, the percentage of tumor initiating cells in melanoma may vary dramatically depending on the applied technique. CSCs from melanoma samples were previously claimed to be very rare at about one cell in a million (Schatton *et al.* 2008, Quintana *et al.* 2008). However, about a quarter of melanoma cells can form tumors in xenotransplantation assays (Boiko *et al.* 2010). Hence, it is possible to conclude that either melanoma cells do not fit to CSC model or most melanoma cells behave like CSC within tumors (Clevers 2011). Similarly, serial xenograft studies in pancreatic cancer suggested that tumor growth is normally not sustained by CSC but rather by transiently activated tumor initiating cells in pancreatic tumors (Ball *et al.* 2017). As these examples have suggested, determining minority subpopulations as CSC may not be relevant in all cancers since not all cancers fit to the frequency dependent CSC model. Previous studies with leukemia were only successful due to less intratumoral heterogeneity than in solid cancers. Coupled with extensive knowledge on cell surface antigens, functional differences within genetically homogenous populations of leukemia cells could easily be determined (Clevers 2011). However, most of the cancer cell behaviors and the surface marker expressions in solid tumors were determined due to cell-to-cell and cell-to-ECM interactions within the tumor microenvironment (Batlle & Clevers 2017). Nevertheless, these inherent differences make it impossible to model such interactions in any transplant experiments. Consequently, due to technical limitations of xenotransplant experiments and the differences in conditions of the microenvironment in spontaneous vs. grafted tumors, these types of approaches were not found to be very effective in identifying CSCs in most cancers.

Lineage-Tracing Experiments

Analysis of tissues through genetic-lineage tracing become the standard for identifying adult tissue SCs in recent years. In general, lineage tracing allows us to determine SCs in solid tissues within living organisms without disturbing the original tissue. Lineage tracing usually relies on finding a marker gene (like Lgr5 in intestinal SCs) which enables specific expression of an inducible recombinase (like Cre recombinase). The latter helps to facilitate stable activation of a reporter gene (like LacZ, GFP or RFP) in the progeny of a desired cell population which can be further tracked and studied due to continued expression of the reporter gene (Barker & Clevers 2010, Kretzschmar & Watt 2012). SC like properties of the progeny cells can be assessed within a given subclone by the use of this approach. In parallel to observations with CSCs, adult SCs behave quite different than transplanted SCs. For instance, in mouse hair follicle SCs, transplanted hair follicle SCs were observed to turn into any epidermal lineages whereas they could only produce hair follicle lineages in lineage tracing experiments (Morris *et al.* 2004). All these findings collectively support that the transplantation-based approaches are not sufficient to understand the fate and potential of adult SCs in situ (Batlle & Clevers 2017). Genetic-lineage tracing-based approaches, on the other hand, may be limited due to the availability of SC markers yet they provide unprecedented amount of information about the true characteristics of SCs and their progeny in organizing tissue hierarchy and architecture.

The value of understanding SC characteristics through lineage tracing is not different in tumors. Therefore, many studies recently focused on identifying CSC markers and tracing CSC behaviors in mouse and human models of spontaneous tumor development and dissemination. The first such study was carried out with a mouse model of

carcinogen induced papilloma, in which SC like clones within tumor were tracked with an inducible basal cell specific Krt14-Cre driver system (Driessen *et al.* 2012). In this model, most of the label retaining cells disappeared since these cells were terminally differentiated and lost their SC like properties. However, some label retaining cells within traced clones kept surviving and produced large flocks of progeny cells in benign growths. In the same study, mathematical models generated from lineage tracing data demonstrated that CSCs may divide symmetrically or asymmetrically in stochastic patterns to give rise to CSCs or progeny cells in a similar manner to adult tissue SCs within normal epidermis. Moreover, similar observations were made for the identification of intestinal CSCs in a different model of mouse adenoma (Schepers *et al.* 2012, Kozar *et al.* 2013). In one study, a mutant floxed Apc gene ($Apc^{fl/fl}$) was specifically induced in cells expressing intestinal SC specific Lgr5 marker by the use of Lgr5-EGFP-Ires-cre-ER^{T2} knock in allele (coding for a cre recombinase in Lgr5 expressing cells upon tamoxifen induction). Upon activation, Apc mutant cells could be observed through the use of tdTomato (red) fluorescent reporter (Schepers *et al.* 2012). After these cells initiated the growth of tumor mass, another inducible reporter gene (R26R-Confetti) was activated via tamoxifen injection which turned Lgr5 positive cells into random colors, in one case some of the red Apc mutant cells turned into blue upon tamoxifen induction and later on it was observed that most of the tumor mass was occupied by these blue cells (Schepers *et al.* 2012). This and similar other experiments provided the support for hierarchical organization of cells within tumors and the presence of only a minor part of Lgr5 expressing neoplastic cells acting as CSCs in intestinal tumors. In a mouse model of breast cancer with MMTV-PyMT, it was shown, by the use of multi-colored lineage tracing via intravital imaging, that some colonies were observed to initially grow but later disappeared while other colonies quickly expanded to become dominant growth within tumors (Zomer *et al.* 2013). These studies nicely illustrate intratumoral cell dynamics and tumor heterogeneity as well as the presence of CSCs in mouse breast tumors.

Some preliminary transcriptomic studies suggested that human colorectal tumors are also organized into subpopulations as in the case of normal epithelia (Merlos-Suarez et al 2011). These studies also provided approaches to analyze CSCs in organoids (Dalerba *et al.* 2011). Later on, other studies put these principles in application involving patient-derived and CRISPR/Cas9 edited organoids, together they solved the problem with inability to model human cancer for lineage tracing and CSC identification. Similar to mouse experiments, xenografts made up of Lgr5 expressing (stem cell like) organoids were observed to generate large amount of progeny cells for longer time before eventually differentiating while xenografts with differentiation marker expressing (like Krt20) organoids stopped growing and usually disappeared (Cortina *et al.* 2017, Shimokawa *et al.* 2017). Therefore, lineage tracing experiments can predict the true nature of CSCs within the tumor microenvironment in both mice and human models of tumorigenesis. These studies also provide strong tools for understanding cancer development and suggesting potential therapeutic applications.

CSC plasticity: Stemness, tumor microenvironment and EMT

CSCs and Stemness

Even though most of the tumors arise from a single cell with unlimited proliferative ability, various functions of tumors from tumor initiation to progression and metastasis are all achieved through ability of the tumor cells to differentiate or de-differentiate into other cell niches by the help of tumor cell plasticity (Marusyk *et al.* 2012, Magee *et al.* 2012). The great level of plasticity of CSCs is the reason behind their ability to adapt to ever-changing and harmful conditions within the tumor microenvironment (Beck & Blanpain 2013). Heterogeneity within tumors is not only due to cellular plasticity but also their ability to alter tumor microenvironment through complex interaction of various cell types like tumor cells, stromal cells and immune cells (Göktuna *et al.* 2018). Therefore, we need thorough understanding of mechanisms leading to tumor cell plasticity to fully appreciate CSC behavior in various cancers. The mechanism of plasticity is governed by several factors within the tumors, such as mutability, stemness and trans-differentiation, epithelial to mesenchymal transition (EMT) and creation of an inflammatory microenvironment (Göktuna *et al.* 2018). All these factors act in a synchronized manner favoring continuous tumor development and metastasis.

Since most CSCs follow the basic rules operated in normal tissues, we start our discussion with plasticity of SCs in normal tissues to understand those mechanisms governing tumor cell plasticity in general. In normal tissues, plasticity is usually due to ability of SCs to change their positions through vertical up or down movements within tissue hierarchy (Batlle & Clevers 2017). Recent advances in lineage tracing have revealed that plastic potential of tissue SCs is much greater and common than previously appreciated. In colonic epithelium, for example, secretory lineage cells or enterocytes can readily shift and replace SC niche upon loss of Lgr5 positive SC compartment (Tian *et al.* 2011, van Es *et al.* 2012, Tetteh *et al.* 2016). Other studies also put forward that upregulation of signaling pathways such as Notch and Wnt from neighboring cells are essential in maintaining a SC niche in colonic crypts (Sato *et al.* 2011). In a more recent study, it was also observed that Gli1 expressing stromal cells can also assist SC differentiation program through expression of Wnt ligands (Değirmenci *et al.* 2018). Therefore, neighboring Paneth cells or enterocytes are not the only factors in their confinement to SC like properties through widely enhancing plasticity within colonic epithelium. In other tissues like trachea and kidney epithelium, similar observations with lineage tracking showed that SC compartment can be

compensated by the other cell within the same tissue (Rock *et al.* 2009, Kusaba *et al.* 2014). Consequently, mechanisms regulating trans-differentiation can quickly adapt different types of cells to changes in tissue microenvironment so that plasticity or stemness is not limited to a given niche (like SCs) but can also be activated in other cell types on a demand basis.

Like in normal SCs, plasticity is not the inherent property of CSCs in tumors. In other words, both CSCs and other tumor cells are plastic and make necessary phenotypic transitions upon activation under suitable environmental cues or signals. This phenomenon has been illustrated in different cancer models of breast, and colorectal cancers (Gupta *et al.* 2011, Schwitalla *et al.* 2013). The first studies were carried in breast cancer cell lines from which different cell populations of cells with SC, basal or luminal-like phenotypes were obtained. It was observed that all these cell populations were able to shift to other phenotypes and repopulate missing niches proportional to stochastic ratios found in the original cell line (Gupta *et al.* 2011). Regardless of these observations, only SC like cells showed tumorigenic abilities fitting to CSC definition. However, when the environmental cues were altered through co-culturing experiment, all these three cell populations could become equally tumorigenic to be able to form xenotransplants efficiently. Hence, we can conclude that breast cancer cells like in normal tissues are not fixed in certain niches and that they may change their phenotypic properties to adapt changing environmental signals to further tumor development.

CSCs and Tumor Microenvironment

Tumor microenvironment is very important in shaping tumor development and is the source for plasticity of tumor cells. Tumor microenvironment shapes every facet of the tumor cell plasticity through interactions between tumor cells in different populations and also their interplay with stromal and immune cells. Signaling machinery activated through interactions of these cells with each other and with the surrounding extracellular matrix (ECM) gives rise to spatiotemporal changes in stemness, de-/trans-differentiation and EMT programs to adapt tumor cells to differing conditions within tumor microenvironment (Göktuna *et al.* 2018). Many studies in colorectal cancer provided support for the importance of CSC functions in tumor cell plasticity. Since Wnt ligand expression in crypts is critical for sustaining an undifferentiated state in intestinal SCs (ISCs), most colorectal tumors are initiated upon constitutive Wnt activation which leads to a cryptic progenitor phenotype in resulting cancer cells. Consequently, supporting bottom-up hypothesis of colorectal tumorigenesis, Wnt-activated ISCs were proposed to be the source of colorectal cancer and all the subpopulation of tumor clones should be coming from these progenitors (Barker *et al.* 2009).

Other studies in colorectal cancer models have suggested that non-CSCs can also form neoplastic growths just like their SC partners. In one such study, it was found that regardless of having the same activating mutations for Wnt signaling, tumorigenic ability of tumor cells was found to be affected by Wnt expression levels in each cell (Brabletz *et al.* 2001). Parallel to these observations, non-CSCs were observed to acquire tumorigenic and self-renewing abilities upon HGF signals from stromal cells (Vermeulen *et al.* 2010). Finally, in our previous work with Wnt-driven tumor models in mice, we have shown that top-down model of tumorigenesis where non-SCs can also generate neoplastic growths in tumor subclones was equally plausible (Schwitalla *et al.* 2013). During colorectal cancer initiation, active-NF-$_{K}$B signaling can initiate a chronic inflammatory microenvironment which can trigger de-differentiation on non-SCs to a more SC like phenotype. In this study, we used an inducible Xbp1-Villin-Cre-ER^{T2} mediated activation of both Wnt (Apc$^{fl/fl}$) and NF-$_{K}$B (Ikba$^{fl/fl}$) signaling specifically in differentiated cells which de-differentiated into a SC like phenotype and promoted neoplastic growths up in the villi (Schwitalla *et al.* 2013). In another study, Elp3 signaling in Dclk1^{+} (Tuft cell specific marker) was found to be essential in maintaining Lgr5^{+} CSC niche. Although Dclk1 was previously identified as a terminal differentiation marker and its absence had no observable phenotype in normal colonic epithelium, Dclk^{-}Lgr5^{+} CSCs or organoids were observed to be largely lacking of regenerative and tumorigenic abilities (Ladang *et al.* 2015).

Studies with human colorectal organoids also further support the importance of the microenvironment driven plasticity in tumor development (Shimokawa *et al.* 2017, de Sousa e Melo *et al.* 2017). In one study with human colorectal cancer organoid xenografts, it was observed that human colorectal cancer cells can behave much similarly to tumor cells in mouse models. An inducible suicide gene (Casp9) was inserted into human colorectal organoids through CRISPR/Cas9 editing (Shimokawa *et al.* 2017). This inducible gene is activated selectively in Lgr5^{+} cells so that SCs are totally eliminated and organoids halted growing. Upon removal of the inducer, organoids resumed growing from remaining terminally differentiated cells. Further studies revealed that terminally differentiated Krt20^{+} cells repopulated Lgr5^{+} niche which supports tumorigenic phenotype in organoid xenotransplants (Shimokawa *et al.* 2017). In a similar study with diphtheria toxin induced Lgr5 ablation in human organoids, removal of diphtheria toxin resumed tumor growth in original tumor but not in distant metastases of the same tumor (de Sousa e Melo *et al.* 2017). Therefore, tumor microenvironment can affect plasticity differently in the original site and the metastatic colonies. However, in other cancers like glioblastoma, plasticity within cell populations was observed to be very limited and no replacement of CSCs niche by other cells was observed in a mouse model of glioblastoma (Suva *et al.* 2014). Therefore, the level of tumor cell plasticity varies greatly depending on the context, tissue of origin,

site of growth and the interactions between different cells within the tumor microenvironment.

Signaling machinery activated through interaction within the tumor microenvironment is also very important for maintaining SC populations for further tumor growth. As tumor cells interact with each other to maintain self-renewal and to enhance their tumorigenic potential, they also interact with other cell types within the tumor microenvironment. Previously, we mentioned about the importance of their interaction with stromal cells which help them to maintain stemness or de-differentiation (Değirmenci *et al.* 2018, Vermeulen *et al.* 2010). Inflammation in the tumor microenvironment is another important component of plasticity. Although we have mentioned that over-activated NF-$_K$B signaling is important for de-differentiation of non-SCs in tumors, hyper-activated NF-$_K$B signaling may also lead to myeloid specific anti-tumor response in a mouse model of IKK$_K$ specific ablation (Göktuna *et al.* 2014). Moreover, IKK$_K$ (NF-$_K$B) ablation in tumor associated fibroblast potentiates them to secrete more HGF which was previously shown to be increasing de-differentiation into SC like phenotype in tumor cells (Pallangyo *et al.* 2015, Vermeulaen *et al.* 2010). Chronic inflammation can still benefit the tumor growth in a cell and context dependent manner as revealed by studies with IKK$_K$ or IKK$_K$ specific ablations in Wnt-driven models of tumorigenesis (Greten *et al.* 2004, Göktuna *et al.* 2016). All these findings illustrate that we urgently need to extend our understanding of tumor cell plasticity not only via tumor specific isolated model but also by finding ways to simulate complex interactions within the tumor microenvironment.

CSCs and EMT

When neoplastic growths reach to a certain size, their further expansion is usually restricted by the surrounding tissues and the basal lamina (Göktuna *et al.* 2018). Then it becomes increasingly difficult to keep the pace of the proliferation due to limiting factors like lack of space, nutrients, and oxygen supply. At this stage, epithelial to mesenchymal plasticity (EMP) helps tumor cells quickly adapt these new conditions, granting them the ability to shift their phenotypes from epithelial to mesenchymal for motility and invasion or back to epithelial phenotype for colonizing distant locations (Kalluri & Weinberg 2009). Although EMT and mesenchymal to epithelial transition (MET) are natural processes during development, growth and wound healing mechanisms, tumor cell may hijack these machineries to provide themselves with many advantages. During the process of EMT, tumor cells constantly acquire new characteristics like anchorage independent growth, orchestrating immune or endothelial cells in immune tolerance or induction angiogenesis, matrix remodeling, vascularization to intravasate into the blood circulation (Kalluri & Weinberg 2009). Most of these changes are related to mesenchymal phenotype since only fibroblast like cells can activate molecular pathways related to phenotypic changes in EMT through alteration of cytoskeleton dynamics and shifts in cell to cell or cell to ECM interactions (Friedl & Alexander 2011). Although mesenchymal state is essential for invasion and motility, it is not suitable for the proliferation of the tumor cells (Mejlvang *et al.* 2007). Epithelial state, on the other hand, increases the efficiency of autocrine and paracrine growth signals due to the densely packed epithelia and helps rapid proliferation of tumor cells both in the primary and in metastatic growths. Therefore, EMP is essential for tumor cells to adapt and change their phenotypes depending on external signals and ever-changing dynamics of the tumor microenvironment (Polyak & Weinberg 2009).

As EMT is associated with migration, invasion, metastasis and chemotherapy resistance, it is not surprising that the relation between CSCs and EMT has been the focus of large amount of studies. As a result of such studies, it has been put forward that EMT also enhances stemness and tumor initiating potential in different cancer cell lines (Puisieux *et al.* 2014). More notably, tumor cells expressing high levels of EMT marker Snail1 were found to have elevated tumor initiating and metastatic capabilities in mouse and human models of breast cancer (Ye *et al.* 2015). Initially, most of such observations were interpreted as the specific induction of EMT in CSC niche, yet later studies have clearly identified that CSCs just have different EMT programs than those operated in other tumor cells (Guo *et al.* 2012). Furthermore, many recent studies have demonstrated that metastatic cancer cells retain their epithelial phenotype in distant locations and even EMT may not be necessary for the metastasis altogether (Nieto *et al.* 2016, Zheng *et al.* 2015). Indeed, many reports support the notion that EMT repression is necessary for metastatic colonization as mesenchymal like cells were found not metastasizing efficiently (Celia-Terrassa *et al.* 2012, Tran *et al.* 2014, Tsai *et al.* 2012, Ocana *et al.* 2012). Additionally, intravital imaging in a mouse model of breast cancer confirmed previous claims with EMP as the tumor cells were observed to undergo MET upon metastasizing to distant organs (Beerling *et al.* 2016). Moreover, other studies have shown that Twist1 and Zeb1 expressions are differentially regulated during the course of tumor initiation, progression and metastasis and intermediary levels of these EMT regulators are required for tumor initiating CSC phenotypes in breast and skin cancers (Beck *et al.* 2015, Schmidt *et al.* 2015, Chaffer *et al.* 2016). Consequently, all the studies presented above have clearly demonstrated that CSC phenotypes are not fixed but highly plastic in nature and subject to change for adapting various conditions in the tumor microenvironment.

Targeting CSCs for Cancer Therapy: Premises and Pitfalls

Most of the chemotherapeutic strategies aiming to target highly proliferating cancer cells usually result in failure due to resistance development. As in many other cases of natural selection, surviving populations cause the

relapse of the tumors with highly chemotherapy-resistant tumor cells (Holohan *et al.* 2010). In most cases, these chemotherapy-resistant cells are found to contain disproportionate fractions of CSCs (Batlle & Clever 2017). Extraordinary chemo- or radiotherapy resistance in tumors may depend on diverse mechanisms provided by upregulation of drug-pumps, enhanced DNA-repair capacity or protection from stress mechanisms (genotoxic stress, ER stress or ROS) in CSCs (Li *et al.* 2008, Diehn *et al.* 2009, Borst *et al.* 2012). Notably, plasticity of CSCs (for tumor development and dissemination) and their ability to adopt a quiescent state (upon stressful conditions) drive drug resistance in many different cancers. Studies in hematologic malignancies have focused on associating hierarchical organization of tumor cells with the drug resistance development (Clarkson 1969). In some pioneering works with leukemia, it was observed that slowly proliferating SCs were the reason for tumor relapse (Clarkson & Fried 1971). Later on, genetic fate mapping experiments in oxaliplatin resistant colorectal tumors demonstrated that tumor relapse after therapy is largely due to populations generated by quiescent CSCs (Kreso *et al.* 2013). Slowly proliferating CSCs in glioblastoma were found to be responsible for temozolomide resistance whereas ablation of these cells in tumors resensitizes glioblastoma to the drug (Chen *et al.* 2012). Similarly, cisplatin resistance results from slowly proliferating CSCs which found to show dormancy due to TGF rich microenvironment in tumor fronts (Oshimori *et al.* 2015). Similar observations in models of bladder, breast and skin cancers also documented that slowly proliferating quiescent CSCs are the reason for drug resistance and tumor relapse after initially successful anti-proliferative therapy (Kurtova *et al.* 2015, Creighton *et al.* 2009). On the other hand, studies of tumor cell populations from highly proliferative tissues like stomach and intestine have demonstrated that SC pool can be regenerated from differentiated tumor cells after chemotherapy (Stange *et al.* 2013, Wei *et al.* 2016). As previously discussed in tumor cell plasticity, differentiated cells in various cancers may be acting as quiescent SCs to drive drug resistance in these cancers.

Understanding of drug resistance mechanisms due to CSCs provided us with better strategies to target tumors. The first successful story came from studies with leukemia which laid the foundation for using anti-CSC therapy in treating cancers (Novak *et al.* 2009). As we mentioned above, most leukemic cells are arrested in a quiescent undifferentiated state. Therefore, it was hypothesized that induction of terminal differentiation with all-trans retinoic acid could be beneficial for sensitizing tumors to chemotherapy. Results were massively successful and all-trans retinoic acid became the standard therapy for treating acute promyelocytic leukemia. The success of this strategy influenced many other studies in developing alternative ways to target CSCs in hematologic and solid malignancies. For example, targeting epigenetic regulators of stemness like Lsd1 or Bmi1 were found to give favorable results abrogating CSC niches in AML or colorectal cancer models, respectively, without causing noticeable side effects (Harris *et al.* 2012, Kreso *et al.* 2014). Even some Lsd1 inhibitors are currently in phase 2 clinical trials for treating AML. As previously mentioned, genetic ablation of CSCs in glioblastoma, squamous cell carcinoma and colorectal cancer prevents tumor growth (Chen *et al.* 2012, Boumadhi *et al.* 2014, Shimokawa *et al.* 2017). These studies are currently being tried for preclinical application. For instance, the use of Lgr5 antibody in combination with cytotoxic chemotherapy (in combination with cetuximab, an anti-EGFR antibody) yielded favorable results in colorectal cancer therapy (Shimokawa *et al.* 2017). Similarly, antibody-drug conjugates targeting Notch signaling successfully eliminated tumor initiating CSCs in a xenograft model of pulmonary neuroendocrine cancer (Yen *et al.* 2015; Saunders *et al.* 2015). Although above strategies exemplify successes in eliminating tumor initiating CSC, it is still a big challenge to target quiescent CSCs in drug resistance. However, in one study with chronic myelogenous leukemia (CML), ablation of quiescent state by targeting Myc inhibitor Fbxw7 rendered CML sensitive to imatinib (Takeishi *et al.* 2013). In a reverse strategy with a model of bladder cancer, Cox-2 inhibitors were used to block the entry of CSCs into a quiescent state which rendered them susceptible to chemotherapy (Kurtova *et al.* 2015). Targeting CSC metabolism also yielded fruitful results in abrogating chemoresistance in models of melanoma, pancreatic and oral cancers (Roesch *et al.* 2013, Viale *et al.* 2014, Pascual *et al.* 2017).

Future Directions

From the early observations of tumor development and dissemination, we started to notice that tumors are composed of cells of different hierarchies reminiscent of the organization in the normal tissues. Tumors from different cancers harbor self-renewing CSC niches which regenerate tumors or turn themselves into various other types of cells within the tumors. By applying the simple principles we have learned from normal SCs and by identifying tumor microenvironment dependent requirements of CSCs in each tissue and cancer separately, we can fashion better therapeutic strategies through modulating CSC behaviors in cancers.

In early studies, allografts and xenografts were crucial for understanding CSC behaviors in the tumors. However, with the advance of more powerful molecular and imaging techniques, lineage tracking became the standard for the identification of CSCs. Coupled to further developments in intra-vital imaging, high-end genome editing tools and the use of organoid models, we learned a great deal of CSC functions in tumor development and dissemination in many cancers. From these observations, we could deduce that SCs are not always rare, dormant or fixed to a certain niche. The great level of variability of CSCs is indeed due to the plasticity of the tumor cells

which provides adaptation of tumors to constantly changing conditions within the tumor microenvironment. The tumor cell plasticity within tumors is forged through adaptations arising from mutations, EMT, and other interactions within the tumor microenvironment. Hence, inflammation and tumor to immune cell interactions are essential components of the mechanisms leading to plasticity.

While not all tumors in every cancer follow the CSC hierarchy or dynamics, there is a great level of plasticity between different populations of cells ultimately leading to great level of heterogeneity within tumors. Besides, the tumor cell plasticity brings about many challenges for our understanding of CSC in tumor development and dissemination. There are still many questions left to be answered by future studies. We still do not know how tumor cells define the CSC niche? We also do not know to which extent the tumor cell phenotypes within tumors can be switched? How do different states of tumor cells affect basic mechanisms in tumor plasticity, EMT or therapy resistance? What are other factors regulating regeneration of lost CSC compartments in tumors? Even more complicatedly, CSC behaviors differ largely in their responses to produce therapy resistance in cancers. Therefore, to answer some of these questions more powerful techniques such as single cell sequencing and multiplexed MALDI-imaging analysis are required to map cell to cell interactions and hierarchical distributions in tumor and in surrounding tissues. The ultimate goal of these studies is to develop superior strategies to tackle cancer. To do so, we need to foster our knowledge on tumor development. Therefore, better understanding of the rules governing hierarchical distribution of the cells and their interactions within the tumor microenvironment will be crucial to reach this goal.

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Review

CONCISE REVIEW: β CELL REPLACEMENT THERAPIES IN TREATMENT OF DIABETES MELLITUS

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Abstract: Metabolic rate of glucose uptake is generally controlled by a feedback mechanism covering islet β cells and insulin-sensitive tissues, wherein tissue sensitivity to insulin influences the level of β-cell comeback. In case of insulin presence, β cells preserve standard glucose tolerance via enhancing insulin production. Even though β-cell dysfunction has a strong hereditary component, environmental alterations carry an important part as well. Current research methods have facilitated to establish the important part of hexoses, amino acids, and fatty acids in the development of insulin resistance and β-cell dysfunction, therefore more operative treatments to slow the progressive loss of β-cell function are required. Latest discoveries from clinical research deliver significant information about approaches to stop and treat diabetes and some of the adversative properties of these interferences. Generation of satisfactory numbers of pancreatic endocrine cells that work in the same way as primary islets is of supreme prominence for the expansion of cell treatments to cure. In this study, we focused on different techniques starting from islet and pancreas transplantations individually and ending on new therapies such as stem cell technology and bioengineering. We aimed to establish a comprehensive and detailed explanation of treatment perspectives for islet cell loss. This review is carrying a novel potential for enlightening the current treatments and future-based therapies.

Key words: Stem cell therapy, islet cells, pancrease.

Özet: Glikoz alımının metabolik oranı, genellikle adacık hücrelerini ve insüline duyarlı dokuları kapsayan bir geri bildirim mekanizması ile kontrol edilir. İnsülin varlığında β hücreleri insülin üretimini artırarak standart glikoz toleransını korurlar. β-hücre disfonksiyonunda kalıtsal bileşenlerin etkileri yüksek olsa da, çevresel değişikliklerin de önemli bir rol oynadığı gösterilmiştir. Güncel araştırma yöntemleri, insülin direnci ve β-hücre disfonksiyonunun oluşmasında heksozların, amino asitlerin ve yağ asitlerinin etkilerinin varlığını göstermekle birlikte, hücre fonksiyonunun aşamalı kaybını yavaşlatmak için daha etkili tedavilerin gerekliliğini de göstermektedir. Klinik araştırmalardan elde edilen sonuçlar diyabetin durdurulması ve tedavi edilmesi ve bu müdahalelerin olumsuz özelliklerinden bazıları ile ilgili önemli bilgiler sunmaktadır. Birincil adacık hücreleri ile aynı şekilde çalışan pankreatik endokrin hücrelerinin yeterli sayıda üretilmesi, iyileştirilmesi için hücre tedavilerinin genişletilmesi büyük öneme sahiptir. Bu derlemede, adacık ve pankreas transplantasyonlarından başlayıp kök hücre teknolojisi ve biyomühendislik odaklı yeni tedavi tekniklerinin incelenmesine odaklandık. Adacık hücre kaybı için tedavi perspektiflerinin kapsamlı ve ayrıntılı bir açıklamasını yapmayı amaçladık. Dolayısıyla bu inceleme, mevcut tedavileri ve geleceğe dayalı tedavileri aydınlatmak için açıklayıcı bir potansiyel taşımaktadır.

Introduction

Diabetes mellitus (DM) is a chronic disease associated with high blood glucose levels and long-term secondary complications characterized by micro-vascular problems such as retinopathy and nephropathy (Silva *et al.* 2006, Petersmann *et al.* 2018). The disease has been conventionally grouped into type I and type II DM depending on the underlying cause. Type I DM is an autoimmune disease caused by the inability of pancreas to produce insulin due to the damage of the insulin secreting β cells, while type II DM is caused by insulin production by pancreas below desired levels or insulin resistance of the body, which is commonly caused by obesity (Akinci *et al.* 2012). Currently, insulin levels are usually maintained with regular injections or continuous infusion of insulin and monitoring of blood glucose levels (Silva *et al.* 2006, Petersmann *et al.* 2018). DM is one of the most prevalent health care problems throughout the world, especially in developed countries. As reported by the World Health Organization (WHO), today 285 million people at minimum suffer from DM. On the other hand,

the projection about the number of people associated with DM in 2030 is 439 million (NCD Risk Factor Collaboration 2016). DM prevalence has gradually improved in the aged population in Europe, regarding to the elevated incidence of obesity. The archieves of the Ministry of Health in Turkey reported that, the rate of DM suffering people was 7.8% in the country by the year 2015. Owing to the excessive quantity of DM patients, Turkey conveys nearly 13% of the cases of Europe (Oksuz *et al.* 2017). The estimated prevalence of the disease worldwide is shown in Fig. 1.

Since the first transplantation performed at the University of Minnesota in 1966, pancreas transplantation has been well-established as the treatment of choice for type I DM. Pancreas transplant numbers steadily increased until the beginning of the 2000s except a decrease observed between the period of 2005-2014. Today, transplantation is accepted as the only modality with clearly-defined indications and long-term insulin free patient survival. These current indications defined by the American Diabetes Association are as follows (Assoc 2006):

1- Patients with end-stage renal disease who had or plan to have a kidney transplant,

2- If there is no indication for kidney transplantation, pancreas transplantation should only be considered for the patients who have the following three criteria:

a) A history of frequent, acute, and severe metabolic complications (hypoglycemia, hyperglycemia, ketoacidosis) requiring medical attention,

b) Clinical and emotional problems with exogenous insulin therapy that are so severe as to be incapacitating,

c) Consistent failure of insulin-based management to prevent acute complications (Assoc 2006).

Impact of pancreas transplantation on patient survival has been controversial since there are no prospective randomized studies evaluating the survival benefits of SPK (simultaneous pancreas and kidney), PTA (pancreas transplant alone) or kidney transplantation. However, registry analysis shows comparable patient survival after SPK in relation to living donor kidney transplantation. It has been shown that secondary complications of DM can be prevented or progression of these complications, such as diabetic nephropathy or retinopathy, can be delayed most likely due to normoglycemic status achieved after successful pancreas transplantation (Fioretto *et al.* 1998).

Transfer of pancreatic islet cells as a possible treatment for type I DM has become the topic of extreme attention over the past two decades. A procedure, identified via the infusion of several new islets subsequent to the host immune suppression with non-steroidal immunosuppressive regimen, has been shown to be effective in severe types of type I DM. Nonetheless, the issue of the worldwide lack of transplant ready islets has yet to be fixed. Furthermore, islet transplantation has been hindered by immune response and repeated attacks towards islets upon fundamental autoimmunity (Halpin *et al.* 2017).

Immunosuppressive routines are effective in stopping islet failure for months to years, therefore these treatments may upsurge the possibility for specific malignancies and unscrupulous infections. Paradoxically, all ordinarily used immunosuppressive medication covering steroids or calcineurin inhibitors have been stated to have opposing effects on pancreatic β cells (Shapiro *et al.* 2000, Hafiz *et al.* 2005). Consequently, these influences encourage strategies to find renewable bases for islet replacement tissue. However, immunological and technical barriers seem to hinder the widespread utilization of this treatment modality (Halpin*et al.* 2017, Ricordi & Strom 2004). The cell therapies for the treatment of islet cell loss between the years 2000 and 2017 are listed in Fig. 2.

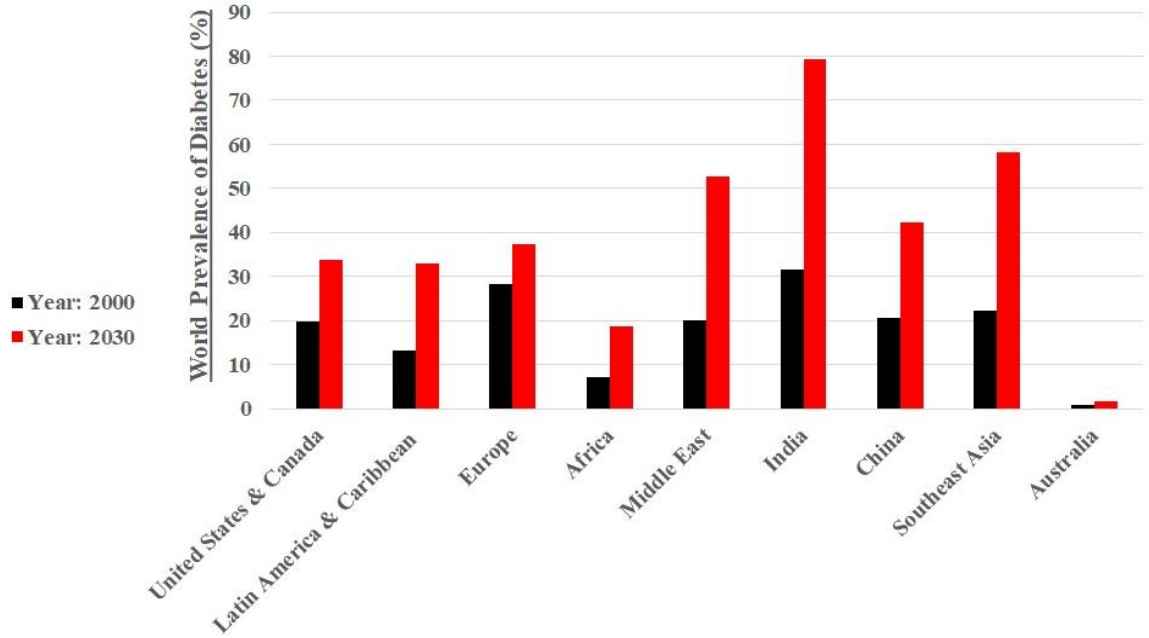

Fig. 1. The worldwide prevalence of DM in 2000 and the estimated prevalance in 2030 (NCD Risk Factor Collaboration 2016).

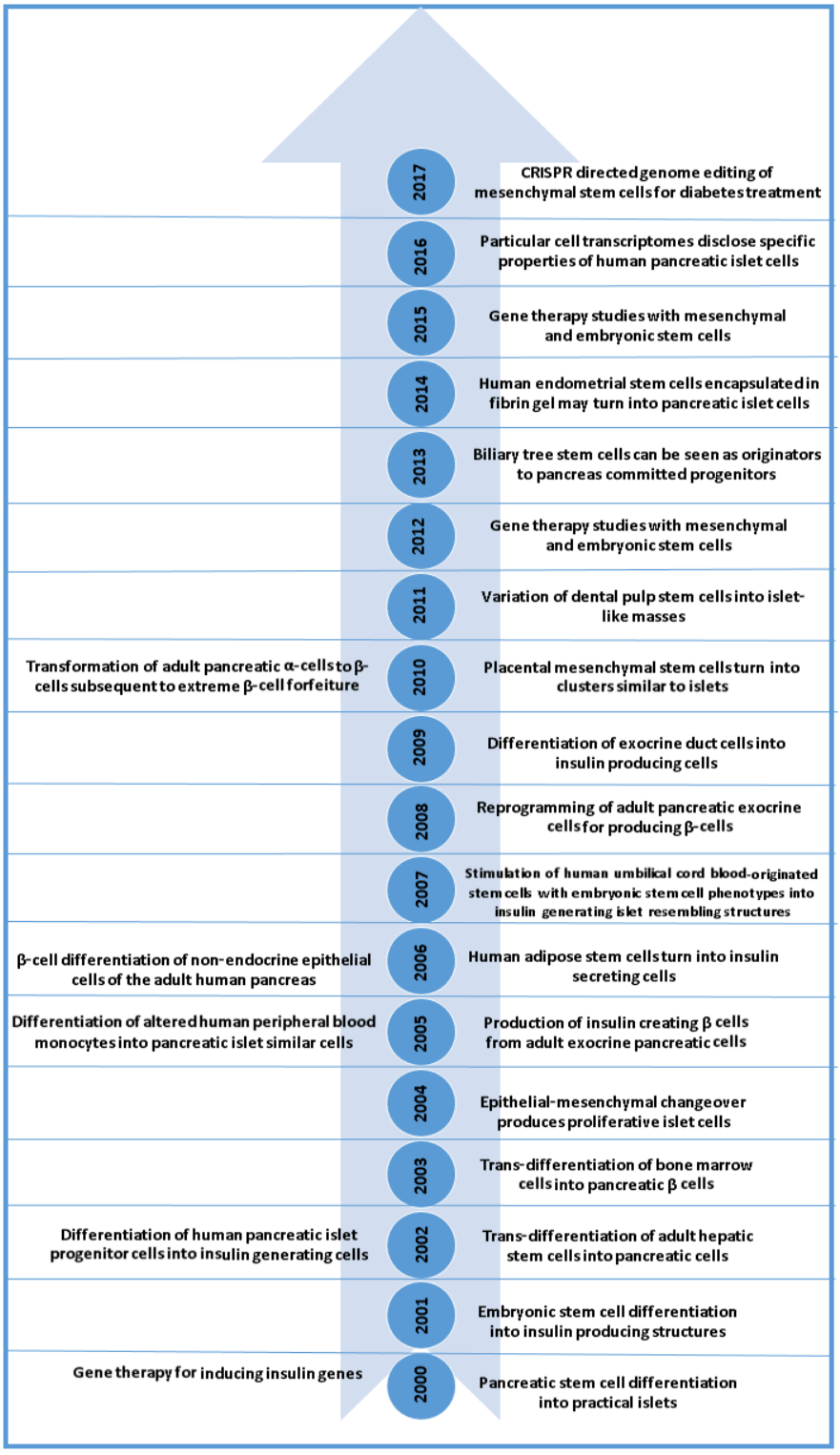

Fig. 2. An overview of the cell therapies for the treatment of islet cell loss between the years 2000 and 2017(Ellis *et al.* 2017).

Animal Models For The Investigation of Pancreatic Problems

The non-obese diabetic (NOD) mouse and bio-breeding (BB) rat are the most frequently utilized animals that naturally progress diseases with resemblances to human type I DM. In NOD mouse, insulitis exists once the mice are 4-5 weeks old, subsequent to subclinical β-cell obliteration and reducing circulating insulin densities. In BB rat, in corporate with the human disease, ketoacidosis is vigorous and deadly unless exogenous insulin is implemented. Other animal models for type I DM are LETL (Long Evans Takushima Lean) rat, New Zealand white rabbit and Chinese hamster (Rees & Alcolado 2005).

Type II DM animal models include Ob/Ob mouse-monogenic model of obesity (leptin deficient), db/db mouse-monogenic model of obesity (leptin resistant), Zucker (fa/fa) rat-monogenic model of obesity (leptin resistant), Goto Kakizaki (GK) rat, KK mouse, NSY mouse, OLETF rat, Israeli sand rat, fat-fed streptozotocin-treated rat, CBA/Ca mouse, diabetic Torri rat and New Zealand obese mouse. For GK rat, similar to human type II DM, an excess of maternal transmission has been reported. The NSY mouse is mainly valuable when investigating age correlated phenotypes. Causes for utilizing KK mouse instead of the GK rat lies in the advantage of the KK mouse in imitating human obesity and easier generation of transgenic alternatives from mice rather than rats (Rees & Alcolado 2005, Seemayer *et al.* 1980).

Pancreas Transplantation vs. Islet Cell Transplantation

Today, pancreas transplantation or islets of Langerhans transplantation are the only alternatives to daily insulin injections or insulin pump (Ikemoto *et al.* 2009). Pancreatic islet transplantation carries a significant advantage of being less invasive and safer for the patients (Matsumoto 2010).

According to Dean *et al*., the risk of secondary complications of diabetes such as neuropathy, retinopathy and atherosclerotic cardiovascular disease have been shown to decrease by the accomplished pancreas transplantations. Nevertheless, the researchers also reported that most of the data come from a single center and include proportionately small patient cohorts and there have been some conflicting research results (Dean *et al.* 2017).

The risk/profit ratio must be prudently assessed in each patient where islet transplantation is reflected, as opposing events are often detected succeeding to islet transplantation, mostly associated with immunosuppressive treatment. Strict receiver assessment is vital for choosing patients appropriate for islet transplantation. Ultimate applicants for islet cell transplantation are patients with unbalanced type I DM and have a past of plain glycemic awareness, in spite of attempts to correct the illness via skilled medical treatment (Dean *et al.* 2017).

A retrospective research reported results from 33 islet transplantation alone (ITA) and 33 PTA receivers. Notably, to emphasize the potential morbidity of PTA strategy, due to graft thrombosis, seven of the PTA receivers (21%) required allograft pancreatectomy in the beginning of the post-transplant span. Considering all PTA receivers, insulin independence is achieved in 25 of 33 PTA patients in comparison with 19 of 33 ITA receivers. The probability for lack of insulin independence was estimated similar for PTA and ITA receivers as P=0.574 (Dean *et al.* 2017).

Stem Cell Therapy

Different research groups are developing methods to substitute the demolished insulin-secreting cells. Pancreatic islet cell transplantation has so far been the only operative procedure to treat type I DM. Nevertheless, lack of a sustainable source of human islet cells limit this treatment from being utilized in diabetic patients (Vanikar *et al.* 2016, Wu & Mahato 2014). Stem cells have self-renewing features that can create numerous cell types in the body. They are established in adult and fetal tissues, nonetheless stem cells with the broadest evolving capacity originate from an initial stage of the mammalian embryo and called embryonic stem cells (ESCs). Embryonic stem cells may be competently persuaded to distinguish into insulin-producing and further cell lines characteristic of the endocrine pancreas and these cell can be assembled to generate practical pancreatic islet-like structures (Millman & Pagliuca 2017, Kim *et al.* 2018, Pysna *et al.* 2018).

Initially, by a cell trapping system, ESCs were efficiently induced to become distinct into pancreatic ß cells (Soria *et al*. 2001). Conversely, this was a complex progression including genetic modification (Soria *et al.* 2001). Afterwards, (Lumelsky *et al*. 2001) planned a five step procedure which persuaded ESCs to turn into insulin-producing islet-like structures without genetic modification. Then, the five step method was used by Peterson *et al*. who discovered that insulin-positive ESC-derived cells absorbed insulin from culture medium instead of generating insulin by themselves (Peterson *et al.* 2017). For this reason, it is crucial to discover unique stimulation factors that could persuade ESCs to turn into pancreatic ß cells more efficiently (Shi *et al.* 2005).

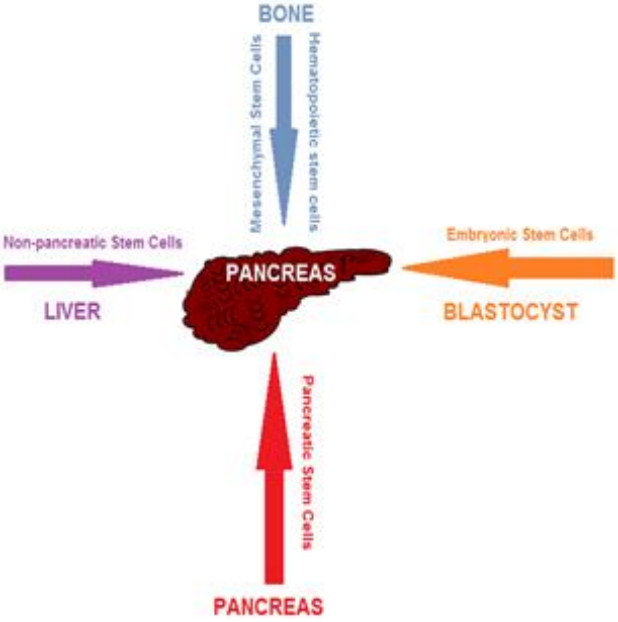

Fig. 3. Different stem cell sources for the islet cell differentiation that has been found so far (McCall *et al.* 2009).

Multipotent stem cells have been defined inside pancreatic islets and in non-endocrine sections of the pancreas. These cells consume the ability of differentiating into pancreatic islet like structure. Fetal stem cells, particularly umbilical cord blood stem cells, consume a benefit, as being an instrument for stem cell

treatment in clinic, mostly due to their low immunogenic potential and their moderately easy receiving source. Yoshida *et al.* (2005) effectively established that the umbilical cord blood stem cells produce insulin generating cells in mice models. Recent researches showed that no rejection occurs even after xenotransplantation of post-differentiated umbilical cord mesenchymal stem cells deprived of immunosuppression treatment (Wang *et al.* 2004).

Studies proposed that pancreatic stem cells carry the ability to turn into liver cells. Therefore, liver stem cells also carry the potential to differentiate into pancreatic cells, especially endocrine pancreatic cells (Yang *et al.* 2002). Some contributing causes which promote absolute endoderm differentiation have been reported. As an example, Activin A, a member of transforming growth factor–beta (TGF-B) superfamily, is a crucial factor which enable mesoderm and endoderm formation throughout gastrulation. All-trans retinoic acid (RA) is a well-defined signaling compound, which contributes to anteroposterior modeling of neuroectoderm and mesoderm in vertebrates (Maden 2001).

In spite of their differentiating effectiveness, differentiation of several stem cells into islet cells has two main problems preventing their clinical application: i) these stem cells are not derived from diabetes mellitus patients, and thus transplanting them might inescapably be rejected by the patients, and ii) the source is not adequate to deliver abundant stem cells. Adult stem cells including bone marrow mesenchymal stem cells and pancreatic stem cells carry, regarding to their inherent drawback, a highly immunogenic feature (Burns *et al.* 2004). Different stem cell sources for islet cell differentiation are listed in Fig. 3.

In order to investigate the factors that might reprogram adult cells into β cells, scientists concentrated on transcription factors, a group of genes augmented for factors that control cell providences through embryogenesis. An in-situ hybridization screen of numerous transcription factors recognized clusters of transcription factors with cell type related expressions in the embryonic pancreas. There is a minimum of 20 transcription factors expressed in mature β cells and their immediate precursors (Sander & German 1997).

Cell Reprogramming & Gene Therapy

There are several methods of gene therapy for DM. The most mutual approach is the transmission of an insulin gene to the liver utilizing a glucose-responsive promoter to deliberate blood sugar adjustment to the insulin transgene. Conversely, the gradual period path of the transcriptional regulation via glucose makes coordinating insulin production with the recurrent undulations in blood glucose levels an exceptionally challenging job (Cheung *et al.* 2000).

Regarding this attitude, the insulin gene paradigm should be altered to generate a gene product, that propagates a single chain insulin or that covers novel cleavage situates that permit a liver protease like furin to manufacture mature insulin from proinsulin. Other methodology of somatic gene therapy is the presentation of transcription or growth factors to the liver to persuade the construction of insulin generating cells in the liver (Kojima *et al.* 2003).

In vivo adult cell reprogramming (transdifferentiation, that is the concept that adult distinguished cells may alter its destiny from one cell type to another) has had diminutive investigational support from mouse models (Thorel *et al.* 2010). Neurogenin 3 (Ngn 3) is recognized as an important transcription factor in the expansion of pancreatic endocrine cells, and the absence of endocrine cells has been established in Ngn 3- deficient mice. An experiment utilizing ESCs where Ngn3 expression was controllable revealed that the expression of genes linked with the pancreatic β-cell growth was upregulated upon expression of the Ngn3 gene. This process induces differentiation and the subsequent insulin-producing cells have been found receptive. Nevertheless, the differentiation effectiveness has been established expressively restricted (Schonhoff *et al.* 2004).

Some studies developed exocrine cells of an adult pancreas as target cells for reprogramming. Exocrine cells originate from pancreatic endoderm as do β cells, and may turn on endocrine programs once detached and cultured in vitro. The transcription factors were carried into the pancreas in adenoviral vectors. It has been revealed that adenovirus specially infects pancreatic exocrine cells, but not islet cells, and since most endogenous β cells exist in islets, fresh formed β cells might be simply identified as extra islet insulin positive cells. The three reprogramming elements, Pdx1, Mafa and Ngn3 were identified to be significant in the embryogenesis of pancreas and β cells. Meanwhile, various extra dynamics are also needed for β cell expansion (Zhou *et al.* 2008). α-cells were at no time thought as a possible basis of cells for β-cell therapy in diabetes. Recent studies showed that the quantity of β-cells originated from reprogrammed α-cells is very variant between people having the similar level of β-cell obliteration. Expression of Pdx1 can be important for the α-cell translation process. Ectopic Pdx1 activity, on its own or joint with other elements, arises hepatocytes or acinar cells into insulin production. α- and β- cells are functionally very parallel, with a related equipment to process glucose and secrete hormones. Expression of Glut2 in insulin generating reprogrammed α-cells, combined with Nkx6.1 and Pdx1, would permit them to discharge insulin upon glucose stimulation same as efficient β-cells (Thorel*et al.* 2010).

One recent study demonstrated a tumor derived K cell (mainly positioned in stomach) which was persuaded to generate human insulin through providing the cells with the human insulin gene associated with the 5' regulatory region of the gene coding glucose reliant insulinotropic polypeptide (GIP) (Cheung *et al.* 2000). Mice expressing this transgene manufactured human insulin especially in

gut K cells. This insulin endangered mice form evolving diabetes and preserved glucose tolerance subsequent to obliteration of the innate insulin generating β cells (Cheung *et al.* 2000).

One study has indicated that, the distribution of a combination of the BETA2 and Btc genes to liver persuaded islet neogenesis and upturned diabetes in mice models (Kojima *et al.* 2003). Another research group has transfected human fetal pancreases with a lentiviral vector which expressed SV40LT in the regulation of the insulin promoter. The transduced pancreases have been embedded into SCID mice afterwards with the aim of turning them into pancreatic tissue. The human β-cells have found differentiated with expressing SV40LT parallel with insulin production, have proliferated and produced insulinomas. The insulinomas have then been incubated with a lentiviral vector that expressed human telomerase reverse transcriptase (hTERT), and the hTERT-transduced insulinoma cells have been attached to other SCID mice to amplify the proliferation of β-cells (Ravassard *et al.* 2011).

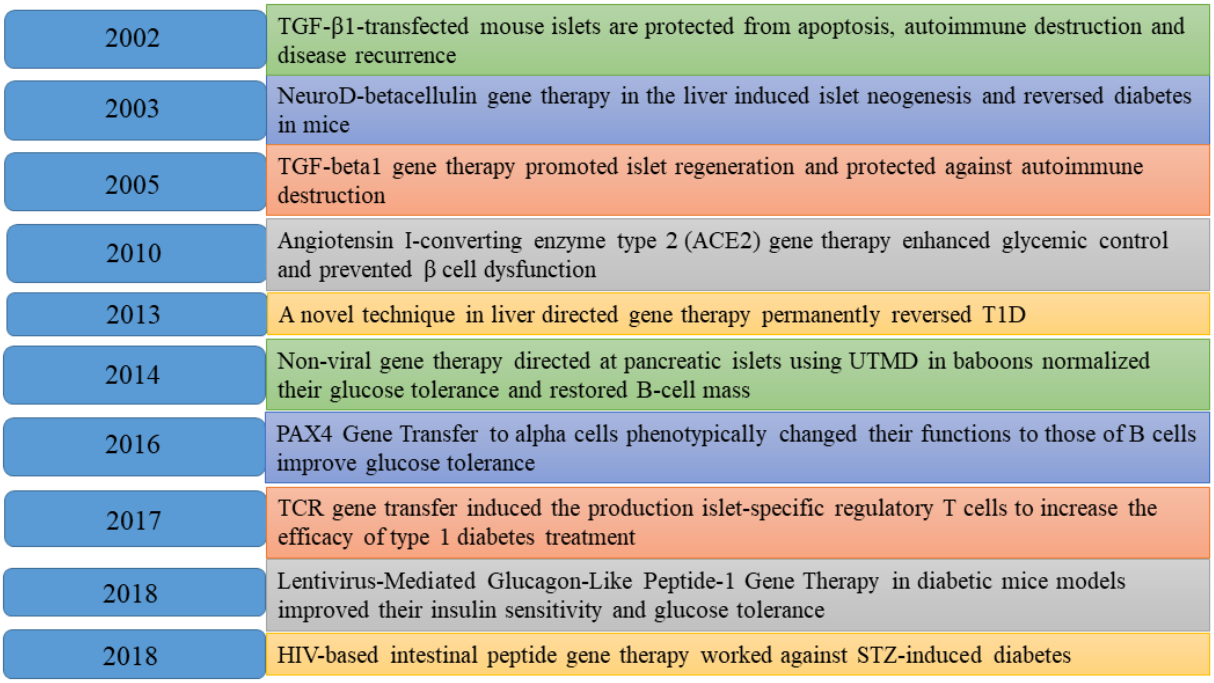

Fig. 4. An overview of gene therapies for the treatment of DM used between the years 2000 and 2018 (Green *et al.* 2018).

Amongst the immunomodulatory mediators, the Th-2 like cytokine IL-10 has been one of the most comprehensively examined and encouraging candidates for operative immune alteration of diabetes treatment. Immune modulation deliberated by adeno-associated virus IL-10 gene therapy has efficiently and intensely enhanced transplant endurance and postponed reappearance of diabetes after islet transplantation in NOD mice (Zhang *et al.* 2003). An overview of the gene therapies for the treatment of diabetes through the years are presented in Fig. 4.

Bioartificial Pancreas

Numerous people around the world agonize from hormone-deficiencies. At present, many of these hormone deficiency diseases may be regulated with systematic intakes of the missing hormone by the patients. Conversely, oral replacement treatment can only postpone the initiation of problems of the illness. At this time, the only certain therapy for these diseases is half-done or total organ transplantation, while the risks elaborated in transplantation are great. A serious risk in organ transplantation is the issue of the host's immune system to reject the transplanted organ. Consequently, receivers will have to use immunomodulating medications for the rest of their lives to evade organ refusal (Kutsogiannis *et al.* 2006).

During the past 30 years, scientists have been trying to improve alternative strategies to stop the requirement of immunosuppressing medication rules after organ transplantation. These so called bioartificial organs on the other hand, mostly cover cells or cell groups inside of a synthetic biocompatible semipermeable membrane that splits the extraneous tissue from the host's immune system (Sander & German 1997). These organs may be implanted in the blood or inserted somewhere in the body via diffusion potential and are meant to entirely imitate the performance and purpose of a healthy organ. Intravascular tools are inserted as an artery-to-vein (AV) shunt in the receiver's body and are commonly originated from tubular hollow fiber membranes. Such extravascular tools may be further categorized in two diverse groups, macro- and micro-capsular devices. Macro-capsular ones can have three key geometries; flat sheet, sealed hollow fibers and macrospheres. These are

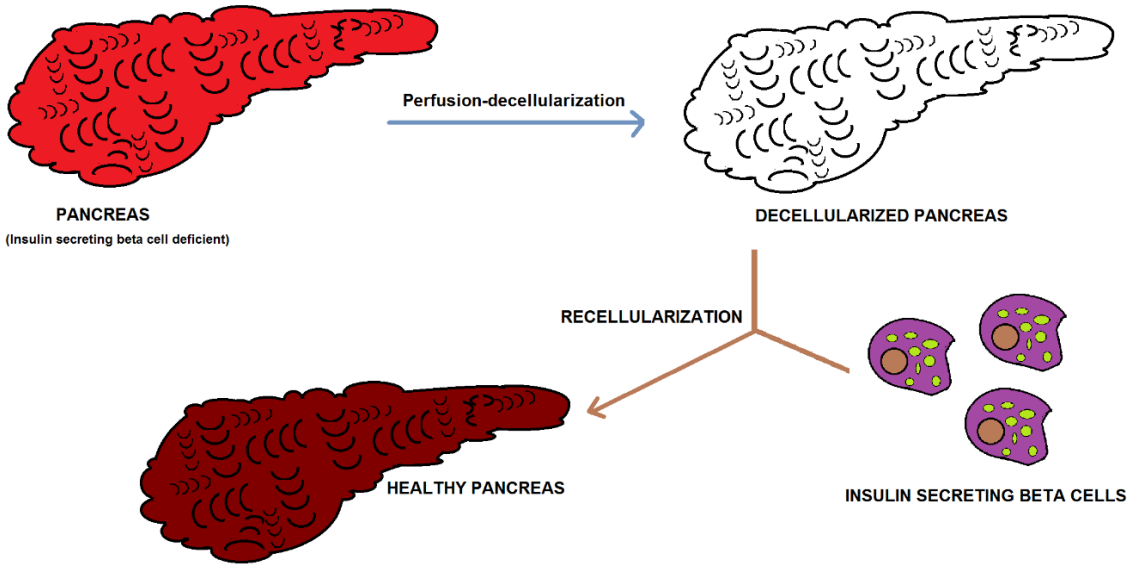

Fig. 5. Tissue engineering of pancreas. The idea is to generate an artificial pancreas. A pancreas is decellularized by perfusion. The subsequent non-immunogenic matrix preserves the organ specific extracellular matrix containing the vascular and ductular proteins. The matrix is then repopulated by mixture of islets to produce a practical and implantable organ (from Song & Ott 2011).

mostly entrenched in the peritoneal cavity but have been inserted in additional areas as well (Li *et al.* 2004, Williams *et al.* 2004). Even though encouraging outcomes have been achieved in early animal studies, the crucial worth of islet encapsulation has been restricted due to 1) deprived biocompatibility of capsule constituents, 2) insufficient immune-isolation regarding to the diffusion of small immune intermediaries, like chemokines, cytokines and nitric oxide, and 3) hypoxia secondary to unsuccessful revascularization (Mirmalek-Sani *et al.* 2013).

Effective islet replacement re-establishes standard glucose uptake in patients with insulin reliant diabetes mellitus (IDDM) then necessitates chronic immunosuppression that is linked with illness and death. The utilization of bioartificial pancreas (BAP) has arisen as a possible choice in clinical islet transplantation regarding to its effectiveness in eradicating immunosuppression. The construction of BAP requires great comprehension of how to preserve the encapsulated islets feasible and operative correctly for elongated periods of time along with facilitating embedding and recovery (Qi *et al.* 2004).

Macro-encapsulation is the envelopment of a huge quantity of islets in a diffusion cavity of a discriminatory membrane for immune-isolation. Microcapsules propose the benefit of embedding and recovery with negligible surgical risk. They can be embedded not only in the peritoneal fissure but also in subcutaneous spot. Different chemical constituents, such as a copolymer of acrylonitrile and vinyl chloride, agarose, alginate, etc., have been utilized as a blockade to avoid refusal of islets (Calafiore *et al.* 2004).

Tissue Engineering of Pancreas

Advances in tissue engineering have facilitated the development of replacement organ tissues for the treatment of injured or degenerative soft tissue. The development of a bioengineered pancreas by appropriate combinations of cells, biomaterial scaffolds and biologically active molecules could provide an alternative avenue for DM therapy (Iacovacci *et al.* 2016). Researchers examined the probability of producing an acellular complete pancreas scaffold via the perfusion, decellularization method and then using this biomaterial as a scaffold to upkeep pancreatic tissue engineering and entire organ regeneration. In the direction of this conclusion, they identified a subsequent native pancreatic ECM scaffold for conservation of ECM arrangement, 3D structural unity and biomechanical features. The conserved ECM scaffold was cyto-compatible, supportive of typical pancreatic cells and showed improved insulin utility once seeded with β cells (Mirmalek-Sani*et al.* 2013).

In a different study, the islets have been grown in chitosan sponge for additional applications. Throughout the process, insulin density of both the interior and exterior of the islet-seeded chitosan sponge have been evaluated. Alterations in the morphology of the islets have been detected. The study also designated that, islets had a moveable presence with an uneven edge, and most were seen as a solitary islet. Infrequently a cluster, involving 2-4 islets changing mostly from 150 to 250 µm in diameter have been detected (Cui *et al.* 2001).

Organogenesis of an endocrine pancreas from transplanted embryonic anlagen was effectively accomplished by Hammerman in 2007. A technique has been established which allows the replacement of developing fetal or neonatal pancreatic tissue that has an amplified capability for pancreatic β-cell growth (Hammerman 2007). Tissue engineering model system is explained in Fig. 5.

Discussion

Regarding the fact that diabetes is an illness that strikes millions of people universally and requires a lifetime inevitability of insulin injections and a high possibility of side effects since glucose blood level regulation is not as good as the physiological glucose regulation. β-cell replacement therapy is extremely necessary. The past 20 years have perceived important improvements in the methodical understanding and management of diabetes. There are two main enterprises on track to fix the β-cell shortage of diabetes: one to produce β-cells ex vivo that are proper for replacement, and the next to induce renewal of β-cells in the pancreas (Mann *et al.* 2009).

The choice of transplanting a pancreas or isolated islets is restricted due to the absence of appropriate organs relative to the great quantity of possible receivers, joint with heavy repercussions triggered by an enduring immunosuppression that has to be weighed contrary to the requirement of insulin injections and it is for that reason solitary suggestible for a subclass of patients with plain medical antiquity (Larsen 2004).

Despite the fact that islet transplantation cannot presently be explained as a complete treatment for DM, the therapy may suggest notable constancy of glycaemic regulation, procuring a cumulative number of patients with continued phases of complete permissiveness from insulin. Preservation of serious hypoglycaemia is the main development that may frequently not be maintained via exogenous insulin treatment (Wagman & Nuss 2001).

New developments in reprogramming through explained genes jointly underline that a restricted amount of factors may program adult cells into another type of cell covering progenitor cells, other mature cells and stem cells. These recent advances based on information of the typical expansion of these cell forms have allowed the alteration of crucial developmental controllers in adult cells (Akinci *et al.* 2012).

A more tempting approach includes the replacement of β cells grown from stem cells, particularly with recent advances in generating large numbers of β cells from human stem cells. Scientific investigations of the safety, admissibility, and efficiency of relocating cells in encapsulated arrangements in type I DM patients are currently in progress (Soria *et al.* 2001).

Regarding the fact that there exist a multitude of methodologies in regenerative medicine including the induction of beta-cell proliferation, reprogramming of other pancreatic or non-pancreatic cells, the differentiation investigation on iPSC, fetal stem cells, or adult stem cells, it is not likely to estimate which technique will appear ahead. Mesenchymal stem cells carry an excessive capacity and are not limited by the ethical problems like embryonic stem cells (Hussain & Theise 2004).

Conclusion

It is understood that a significant dissimilarity exists amongst autoimmune diabetes that is curable with insulin, and serious diseases. Before the cell therapy, the risks and benefits should be accurately considered. For instance, continuing management with immunosuppressive mediators might result in an augmented degree of malignancies. Therefore, stem cell technology and tissue engineering approaches may hold the solution to treat diabetes.

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Review

LIVER CANCER STEM CELLS

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Abstract: Recent studies have shown that many tumour are containing small population of stem cell-like cells, in other words, cancer stem cells. The rapid development of the cancer stem cell field has allowed the scientists and the clinicians to focus on another challenge, which targets cancer stem cells for new therapeutic strategies. Studies showed that novel therapeutic approaches on the selective targeting of cancer stem cells might be extremely successful for prevention of invasion, metastasis, and the relapses of tumors.

Liver cancer is the 5th most common cancer type. It has also been reported as the 3rd most common cause of cancer related death. Recently, progression of hepatocellular carcinoma is thought to be driven by cancer stem cells. There are tremendous efforts to clarify the features of liver cancer stem cells, to identify them within other tumor cells, and finally to develop the new therapeutic models that target those cells. This review summarizes the features of liver cancer stem cells, and its importance for therapeutic approaches.

Key words: Liver, cancer, stem cell, hepatocellular carcinoma.

Özet: Son yıllarda yapılan araştırmalar, birçok tümörün az miktarda da olsa kök hücre benzeri özellik içeren hücreler, bir diğer değişle kanser kök hücreleri içerdiğini göstermektedir. Kanser kök hücreleri ile ilgili araştırmalar ve buna bağlı ortaya çıkan hızlı gelişmeler, gerek hekimlerin gerekse bilim insanlarının diğer bir konu olan yeni tedavi stratejileri için kanser kök hücrelerinin kullanımı üzerinde yoğunlaşmalarına olanak tanımıştır. Bu alandaki araştırmaların sonuçları, kanserin invazyonu, metastazı ve tekrarlamasını önlemede kanser kök hücrelerini hedef alan tedavi yaklaşımlarının son derece başarılı olabileceğini ortaya koymaktadır.

Karaciğer kanseri, tüm kanser türleri arasında 5. sırada yer almaktadır. Kansere bağlı ölümlerde ise 3. sırada yer almaktadır. Hepatosellüler karsinomanın ilerlemesinde kanser kök hücrelerinin çok önemli rolü olduğu gösterilmiştir. Karaciğer kanser kök hücrelerinin tanımlanması, diğer tümör hücrelerinden ayrılabilmesi ve bu hücreleri hedef alan yeni tedavi şekillerinin geliştirilebilmesi konularında çok önemli gelişmeler vardır. Bu derlemede, son yıllarda bu alanlarda yapılan çalışmalar esas alınarak karaciğer kanser kök hücrelerinin özellikleri ve bu hücreleri hedef alan yeni tedavi yaklaşımları özetlenmektedir.

Introduction

Cancer stem cells (CSCs), a small population of stem cell-like cells in tumour- (are extremely resistant to chemotherapy and radiation, and considered as responsible for invasion and metastasis of the cancer. Translational studies present more clues related to the responsibility of these cells for the relapses of tumors (Alison 2005, Kitisin *et al.* 2007).

Liver cancer is the 5th most common cancer type all around the world, and was reported as the 3rd common deadly cancer. According to the data base of Turkish Health Ministry, the incidence of liver cancer was 1.5 to 4.4/100.000 in Turkey between the years 2010 and 2014.

There are tremendous efforts to clarify the features of liver CSCs, to identify these cells within other tumor cells, and finally to develop new therapeutic models targeting these cells. Liver cancer stem cells (LCSCs) have been reported in multiple subtypes of hepatocellular carcinoma. They are considered as the master regulators of hepatocellular carcinoma initiation, tumor metastasis and progression. The stem cells and progenitor cells are elevated in chronic liver diseases (Roskams 2006, Kitisin *et al.* 2007).

This review summarizes the recent findings related to LCSCs, and developments about therapeutic approaches targeting these cells.

The origin of LCSC is still controversial. They are proposed to have two origins, one endogenous and the other exogenous. When LCSCs are intrahepatic, they are considered to have the endogenous origin. Intrahepatic stem cells are mainly localized within the canals of Hering and also seen around the interlobular bile ducts. They turn to hepatic progenitor cells which have short term proliferative capacity.

There are also extrahepatic stem cells of exogenous origin which are derived from bone marrow and/or peripheral blood cells. These exogenous cells have long term proliferation capacity (Alison 2005). Castelli *et al.* (2017) reported that the mutation of cancer cells causes formation of new LCSCs.

How to identify LCSCs?

A. Functional Properties of LCSCs

There are assays that are routinely used to discriminate CSCs from non-stem cells (non-CSCs) on the basis of their functional properties, such as aldehyde dehydrogenase (ALDH) activity, side population analysis, colony formation, sphere formation, and therapy resistance assays (Islam *et al.* 2015, Aktas & Tok, 2014, 2015, Xiang *et al.* 2016, Tok & Aktas, 2017).

ALDH is the main enzymatic system responsible for the clearance of acetaldehyde from the hepatocytes in the liver tissue. It is involved in retinoic acid signaling pathway known as differentiation of the hepatocytes (Tomita *et al.* 2017). ALDH1-expressing cells may serve as a useful differentiation biological marker for hepatocellular carcinoma rather than as a CSC marker. These cells show features of differentiated cells that look like mature hepatocytes but not of CSCs. Taken together, these findings suggest that increased ALDH1 expression is associated with a factor indicative of a well-differentiated morphology and favorable prognosis in hepatocellular carcinoma (Sun *et al.* 2016).

The presence of active transmembrane ABC transporter family members, such as multidrug resistance transporter 1 (MDR1) and ABCG2, can facilitate the efflux of DNA-binding dyes such as Hoechst 33342 in cells with (cancer) stem cell activity known as the side population. Side population cells possess high proliferation potential, tumorigenicity, and anti-apoptotic properties.

B. Cell surface markers

LCSCs can be identified by using cell sorting methods like flow-cytometry and magnetic-activated cell sorting (MACS) separate CSCs from non-CSCs on the basis of different cell surface and intracellular molecules. The most common cell-surface markers used to detect the LCSCs were detailed below (Chiba *et al.* 2009, Yamashita *et al.* 2013, Islam *et al.* 2015).

CD133

Many studies have been performed about the expression of CD133 in different types of cancers, including hepatocellular carcinoma (Sun *et al.* 2016). In human hepatocellular carcinoma cells and cell lines, specifically $CD133^+$ cells, not $CD133^-$ cells, have the ability to self-renewal, create differentiated progenies and form tumors (Ma 2007). They have characteristics similar to those of progenitor cells including the expression of stemness genes. This coincided with the expression of genes associated with stem/progenitor status, such as b-catenin, NOTCH, BMI and OCT3/4. CD 133^+ cells possess a greater ability to form tumors in vivo. When compared to $CD133^-$ cells, $CD133^+$ cells isolated from the cell lines showed higher expression of CD44 and CD34, but both CD133 subpopulations displayed similar expression for CD29, CD49f (integrin a6), CD90 and CD117 (Ma 2007).

Increased CD133 levels are correlated with increased tumor grade, advanced disease stage, shorter overall survival, and higher recurrence rates. Regulation of cell membrane topology is related to existence of CD133.

EpCAM (CD326)

EpCAM is a cell adhesion molecule. In addition to epithelial cells, stem cells also express EpCam (Trzpis *et al.* 2007). It has crucial roles for cell migration and signaling. $EpCAM^+$ cells were correlated with tumor progression and invasiveness. It can mediate cell to cell contact, transmit the signals from the plasma membrane to the nucleus in order to regulate gene transcription (Munz *et al.* 2009). Expression of EpCAM is associated with a stem cell phenotype and regenerative capacity of the cells. DeBooer *et al.* (1999) showed that EpCAM expression is only found in regenerating cells, like hepatobiliary stem cells and progenitor cells. $EpCAM^+$ and Alpha feto protein (+) cells have features of hepatic stem/progenitor cells. EpCAM was also identified as a direct transcriptional target of Wnt/β-catenin signaling pathway in hepatocellular carcinoma (Yamashita *et al.* 2009). Wnt/β-catenin signaling pathway has a crucial role in induction of C-myc, Nanog, Klf4, Sox2 and Oct4 (Zhang *et al.* 2012, Tetreault 2016, Xu *et al.* 2016, Zhan *et al.* 2017)

Oct4

Oct4 hepatocellular carcinoma cells have dysfunctional TGF-β signaling. This stem cell marker is responsible for the regulation of stem cell identity and cell fate. They are likely cancer progenitor cells that have the potential to give rise to hepatocellular carcinoma (Yuan *et al.* 2010, Oishi *et al.* 2014).

Accumulated evidence from various cancer types strongly support the hypothesis that hypoxia sustains the self-renewal characteristics of a portion of cancer cells in hypoxic niches mainly due to the upregulation of Oct4, NANOG, SOX2, Klf4, and c-myc (Mathieu *et al.* 2011, Muz *et al.* 2014).

CD44

CD44 is important for cell adhesion, migration, cell-cell interactions and cell signaling. Mima *et al.* (2012) showed that the patients with hepatocellular carcinoma were characterized with poor prognosis when $CD44^+$ cells regulated the TGF-β-mediated mesenchymal phenotype. Overexpression of CD44s promoted tumor invasiveness and increased the expression of vimentin, a mesenchymal marker, in hepatocellular carcinoma cells.

CD90

Cancer cell behavior related to cell to cell adhesion and signal transduction is affected by the existence of CD90. The higher $CD90^+$ cell proliferation ability and higher tumor promoting capacity observed in vitro correlate with the in vivo data. $CD90^+$ molecule was found to be increased during the progression of hepatocellular carcinoma (Sukowati *et al.* 2013). It has also been reported that $CD90^+$ cells enhanced the motility of $EpCAM^+$ cells when co-cultured in vitro through the activation of transforming growth factor beta (TGF-β) signaling, whereas imatinib mesylate suppressed TGFB1 expression in $CD90^+$ cells as well as $CD90^+$ cell-induced motility of $EpCAM^+$ cells (Yamashita *et al.* 2013).

CD24

Qui *et al.* (2011) demonstrated that hepatocyte progenitor cells possess CD24. It has also been stated that $CD24^+$ liver tumor-initiating cells drive self-renewal and tumor initiation through STAT3-mediated NANOG regulation (Lee *et al.* 2011-a and b).

β-catenin

This is another important marker for cell adhesion and effects the behavior of stem cell in terms of the cell behavior related to epithelial-mesenchymal transition.

CD13

CD13 is involved in regulation of peptides and lipid turnover, and reduction of DNA damage induced by reactive oxygen species (ROS). $CD13^+$ cells predominated in the G_0 phase of the cell cycle and typically formed cellular clusters in cancer foci. Mechanistically, CD13 reduced ROS-induced DNA damage after genotoxic chemo/radiation stress and protected cells from apoptosis (Haraguchi *et al.* 2010).

Ov6

Yang *et al.* (2008 and 2014) showed that Wnt/beta-catenin signaling contributes to activation of normal and tumorigenic liver progenitor cells and $OV6^+$ tumor-initiating cells contribute to tumor progression and invasion in human hepatocellular carcinoma.

Relationship Between LCSCs and Signaling Pathways

The following signaling pathways are considered as stem cell activators. They expedite tumorigenesis.

i. Wnt/β-catenin Signaling Pathway

Wnt pathway is crucial for specification of cellular fates and movements. This pathway is essential for cell development, growth, survival, regeneration and self-renewal. Disruption of this pathway may cause cancer. One third of liver cancer cases contain mutational/non-mutational changes in this pathway (Ishizaki *et al.* 2004).

The pathway diversifies into two branches as β-catenin dependent and non-dependent ways. The β-catenin dependent pathway has critical roles during embryogenesis, while the independent branch is important for adult tissue regeneration (Bowen *et al.* 2008). Yang *et al.* (2008) demonstrated that the fraction of tumor cells expressing OV6 is enriched after Wnt pathway activation, whereas inhibition of β-catenin signaling leads to a decrease in the proportion of $OV6^+$ cells.

ii. SALL4 Signaling Pathway

SALL4 is considered as an oncofetal gene. It is expressed at high levels in fetal liver progenitor cells. It is important for the maintenance of stemness of embryonic stem cells. SALL4 may be a prognostic marker of liver cancer and an indicator of stem cells, playing roles in 5-FU resistance and growth of cells, and tumors with suppressed SALL4 results in differentiation and delayed tumor growth. It has been recognized as a marker for poor prognosis (Sun *et al.* 2016).

iii. TGF-β Signaling Pathway

TGF-β is unique in that in normal cells it acts to maintain the epithelial nature of cells whereas in transformed cells, it is mostly oncogenic in its activity. TGF-β family plays a vital role in proliferation and cellular differentiation in both stem cells and cancer cells. It inhibits cell proliferation and promotes tumor cell invasion by inducing epithelial mesenchymal transition (EMT). Yuan *et al.* (2010) showed the relationship between Oct4 and TGF-β family. Additionally, Fan *et al.* (2014) demonstrated that tumor associated macrophages sustain $EpCAM^+$ LCSCs through TGFβ1 induction of EMT.

iv. Notch Signaling Pathway

This signaling pathway is crucial during liver embryogenesis, bile duct formation, and angiogenesis. $CD133^+$ cells express more Notch. Notch 1 functions as a tumor suppressor. However, Notch 3 is highly expressed in hepatocellular carcinoma.

v. Sonic Hedgehog Signaling Pathway

Mutations and/or aberrant activation of Sonic Hedgehog Signaling Pathway may cause cancers. It controls cell fate specification and pattern formation during embryogenesis. Sonic is the predominant isoform in the liver and up to 60% of human hepatocellular carcinoma express Sonic. Activation of Hedgehog Pathway is critically related to LCSCs and EMT (Villavicenci *et al.* 2000, Lum *et al.* 2004).

Epigenetic Regulation of LCSCs and Progenitor Cells

Although various genes have been identified as stem cell related, the control of stem cells is likely to arise from an integrated expression pattern of multiple genes involved in proliferation and differentiation rather than decimal gene expression (Koike *et al.* 2012).

Anticancer Therapies Targeting Liver Cancer Stem Cells

LCSCs clearly have complex pathogenesis. Targeting the single pathway or a molecule related to LCSCs may

have limited benefits. Combinations of therapies may be needed to eradicate the cancer. Many new therapeutic strategies targeting LCSCs at various stages of differentiation or targeting microenvironment have been attempted. The followings are the recent strategies to eradicate the cancer that target the LCSCs.

i. Blockage of LCSCs Signaling Pathways

Attribution of these signaling pathways are needed to eradicate these resistant cells. Additionally, chemical intervention or downregulation of the signaling pathways may increase the sensitivity of LCSCs to treatment. The followings are the main results of several groups of scientists who demonstrated the importance of the therapies that target LCSCs;

a- Inhibition of Wnt/β-catenin Signaling Pathway causes decreased proliferation and increased apoptosis (Zeng K *et al.* 2007; Gedaly *et al.* 2014).

b- Suppression of Sonic Hedgehog Patwhway in hepatocellular carcinoma decreases cell proliferation, induces apoptosis and the cells are more chemosensitized to 5-fluorouracil (Wang *et al.* 2008).

c- Morell & Strazzabosco (2014) presents evidence related to targeting Notch signaling as a new therapeutic option.

ii. Differentiation of LCSCs

There are preliminary evidences of induced differentiation to effectively deplete tumorigenesis by the tumor initiating cells (Lin 2014). You *et al*. (2014) showed that knockdown of BC047440 induces differentiation of hepatocellular CSCs and should be considered as an alternative treatment. Hepatocyte nuclear factor-4α induces differentiation of LCSCs, which may help to loose of the property of self-renewal (Yin *et al.* 2009). Interferon alpha accelerates hepatocytic and biliary differentiation of oval cells (Lim *et al.* 2006). Oncostatin M, an interleukin 6 related cytokine, induce differentiation of hepatoblasts into hepatocytes.

iii. Targeting EpCAM

Oncostatin M may also be used to induce differentiation and active cell division of dormant $EpCAM^+$ LCSCs.

iv. Detection and Isolation of LCSCs

These therapeutic approaches are based on identification of cell surface molecules on LCSCs and then isolation of the cells (Moghbeli *et al.* 2014). Bach *et al*. (2013) demonstrated the elimination of $CD133^+$ cells. Waldron *et al.* (2014) presented that a bispecific EpCAM/CD133-targeted toxin is effective against carcinoma. Song *et al.* (2013) reported the way to capture the CSCs during circulation. A recent discovery by Lee *et al.* (2011-b) showed that lupeol, a phytochemical present in fruits and vegetables, could target $CD133^+$ liver CSCs by inhibiting their self-renewal and tumorigenic capacity.

v. Microenvironment is crucial for the functions of LCSCs.

Modification of the microenvironment is extremely important for determination of the LCSCs fate. The hepatic microenvironment is drastically altered in chronic liver diseases with the expansion of hepatic progenitor cells. There are approaches that target extracellular matrix, immune cells, the cells in stroma and also cytokine network to eliminate CSCs in the tumor niche.

Activation of stromal cells may induce various signaling pathways, including cytokines such as Wnt, FGF, PDGF, VEGF and TGF-β, and promote the development of LCSCs (Yamashita & Wang 2013). Lin *et al*. (2009) was successful in their experimental study by using a STAT3 inhibitor - NSC 74859- to disrupt TGF-beta signaling in cancer.

Infiltrating lymphocytes in the microenvironment might cause inflammation with the release of free radicals, cytokines, and chemokines, resulting in DNA damage, cell proliferation, and migration (Budhu *et al.* 2006, Hussain *et al.* 2007).

Our studies also demonstrated the relationship between LCSCs, EMT and Type I collagen in the microenvironment (Isan *et al.* 2018).

vi. Inhibition of Epithelial Mesenchymal Transition

The association between CSCs and EMT has recently been established (Mani *et al.* 2008, Chang *et al*. 2015). Sun *et al.* (2014) demonstrated that induction of EMT correlated with the enhancement of CSC marker CD133. Mitra *et al.* (2015) identified the existence of Vimentin on the surface of liver CSCs and utilized this in a separation technique to enrich EMT-positive CSCs directly from primary tumor cells. It has also been shown that CD44 protein levels were enhanced by TGF-β1 treatment and that synergistic interactions between CD44 and TGF-β1 induced EMT and CSC phenotypes through Akt/GSK-3β/β-catenin signaling axis in HCC (Park *et al.* 2016).

The link between CSC and EMT markers has been explored in HCC patient cohort (Zhou *et al.* 2015). Liu *et al.* (2015) demonstrated that shRNA mediated CD44 or CD133 knockdown reversed the EMT phenotype. Similarly, it has been reported in another study that CD44 inhibited the metastasis of hepatocellular carcinoma by reversing EMT (Gao *et al.* 2015).

Taken together, experimental and clinical studies support the hypothesis that CSCs mediate metastasis by maintaining plasticity to transition between epithelial or mesenchymal states. One potential approach is to use monoclonal antibodies, like CD44 or CD133, to target the CSC cell surface antigens that regulate EMT. Targeting of the TGF-β and/or BMI-1 that are involved in EMT might be another promising approach to eradicate these cells.

Future Directions

Liver cancer (hepatocellular carcinoma) is the 5th most common cancer type. It is the 3rd deadly cancer all around the world. From the clinical view, combined therapies, that also target the liver cancer stem cells, might play the key role for prevention of invasion, metastasis, and also the recurrence of the hepatocellular carcinoma. Recent studies suggest that the targeting of single molecules or pathways might have limited benefit for treatment of cancer (Yamashita & Wang 2013). In addition to the direct control of liver CSCs, many other factors are needed for CSC maintenance including angiogenesis, vasculogenesis, invasion and migration, hypoxia, immune evasion, multiple drug resistance, and radioresistance. The promising results of the recent studies give hope to clinicians and the patients for better therapies.

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Review

CURRENT STATUS OF GERMLINE STEM CELLS IN ADULT MAMMALIAN OVARY

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Abstract: The evolutionary and biological mechanisms underlying female reproductive aging have long been a matter of interest. Reproductive biologists have tackled with the relatively limited fertile period in female lifespan compared to male fertility that continues until the late ages. For more than five decades, it has been believed that females are born with a fixed number of germ cells that constitute ovarian follicle reserve and depletion of this reserve causes menopause. However, researchers recently reported findings that support the presence of oogenesis in postnatal mammalian ovaries, which caused a paradigm shift in our current knowledge of reproductive biology. In this minireview, we provide a brief history of one of the central dogmas in reproductive biology and subsequently present recent studies on the existence of germline stem cells in the mammalian ovary.

Key words: Ovary, stem cells, female germline, fertility preservation.

Özet: Dişi üreme sistemi yaşlanmasının altında yatan evrimsel ve biyolojik mekanizmalar uzun süredir ilgi çeken bir konu olmuştur. Üreme biyolojisi konusunda çalışan biyologlar, erkeklerde ileri yaşlara kadar devam eden fertilite ile karşılaştırıldığında dişi yaşamında görece olarak sınırlı bir dönemde görülen fertilite konusunu aydınlığa kavuşturmaya çalışmaktadırlar. 50 yılı aşkın bir süredir dişilerin ovaryan folikül rezervini oluşturan sabit sayıda germ hücresi ile dünyaya geldiklerine inanılmaktadır. Ancak, son dönemde araştırıcılar memeli ovaryumlarında doğum sonrası oogenez varlığını destekleyen ve üreme biyolojisi ile ilgili günümüz bilgilerimizde değişime yol açan bulgular elde etmişlerdir. Bu derleme çalışmasında, üreme biyolojisindeki santral dogmalardan bir tanesi ile ilgili kısa bir tarihsel bilgi verilmiş ve memeli ovaryumlarında germ hattı kök hücrelerinin varlığı ile ilgili son dönem çalışmaları sunulmuştur.

Introduction

Adult stem cells or somatic stem cells with the ability to differentiate into certain mature cell types and regenerate the hosting tissue have been identified in several organs including brain, bone marrow, skeletal muscle, blood vessels, gut, and liver in the human body (Turksen 2014). These cells generally reside inactive for long periods in the tissues until they are needed to produce new cells to maintain tissue function and health.

The idea of the existence of stem cells that can produce mature oocytes in adult ovaries has been a debated concept with several supporting and against evidence. In the male reproductive system, testis hosts germline stem cells that continuously produce sperm cells throughout the reproductive lifespan until the late ages (Brinster 2007). On the other hand, in the female reproductive system, ovaries are known to be constituted with a limited number of primordial germ cells which is referred to as ovarian follicle reserve. During embryologic development, mitotically active oogonial cells rapidly proliferate and colonize the ovary with an approximate population of one million primordial germ cells at birth. This ovarian reserve gradually declines with age due to follicle apoptosis and reduces to ~ 25.000 primordial follicles by the age of 37 years. In the following years, follicle apoptosis accelerates and almost exhausts at the age of 51 years leading to the cessation of the menstrual cycle and menopause (Broekmans *et al.* 2009).

For several decades, it has been a dogma in reproductive biology that females are born with a limited pool of oocytes that maintains reproductive capacity until the time of menopause. However, this dogma of unrenewable ovarian follicle reserve has been recently challenged by several studies suggesting the existence of germline cells with stem cell capabilities in the adult ovaries. In this review, we summarize our current knowledge and understanding of ovarian germline stem cells or oogonial stem cells under the light of evidence supporting and against the case.

Stem Cells in Adult Mammalian Ovary

The debate on follicle reserve in human ovary goes back to the 19th century when Waldeyer (1870) stated that oocyte production ceases at or shortly after birth. Waldeyer's statement was fundamentally based on the results from the studies conducted on ovaries from various mammalian species including dogs and cats. This statement was later supported by Pearl & Schoppe (1921) and finally by Zuckerman's report in 1951, which lead to the conclusion that there was no evidence of postnatal oogenesis in human ovaries. Their conclusion generated a wide acceptance among reproductive scientists and rapidly became a central doctrine in the field of female reproductive biology. Though, during the following period, a few number of studies by Ioannou *et al*. (1967) and David *et al*. (1974) reported the potential presence of stem cells in the postnatal ovaries of primates. In these studies, researchers investigated the presence of oogonia undergoing cell division and found oocytes at various stages of meiosis. Based on the results, they concluded that oogenesis occurs in postnatal ovary. However, their findings were mainly limited to histological evaluation of the tissue sections without any specific markers for oocytes. Despite these early reports, the dogma has remained unchallenged for more than fifty years until recently by the works of Tilly's group from Harvard Medical School (Johnson *et al.* 2004).

In 2004, Tilly's group published a landmark paper claiming the existence of germline stem cells with the ability to produce oogonia and renew follicle pool in postnatal mammalian ovaries (Johnson *et al.* 2004). In their study, researchers initially investigated the follicle dynamics in C57BL/6 mice ovary. By demonstrating the discrepancy between the high follicle atresia rates and the number of follicles observed in the ovary throughout the reproductive period, the authors emphasized that the ovarian reserve would deplete by the young adulthood unless the follicle pool were replenished by an oocyte source, most likely by germline stem cells. To confirm that their findings were not related to the mice strain, they repeated the same experimental design in other mice strains as well including CD1 and AKR/J and reported similar findings. Subsequently, they showed the presence of proliferating germ cells histologically using double-immunostaining for a germ cell marker (MVH) and a proliferation marker (5-bromodeoxyuridine). To show the presence of meiotically active germ cells, researchers investigated synaptonemal complex protein 3 (SCP3), a meiosis-specific protein needed for the formation of axial, lateral elements of the synaptonemal complex, together with *Spo11* and *Dmc1* expressions in juvenile and young adult mouse ovaries. They reported an expression level of 6-25% compared to the expression in testis, which was significant. As a next step, researchers depleted specifically the germline cells using busulphan and examined the change in the follicle reserve. They found that despite the elimination of non-meiotic germline cells, the follicle reserve was stable over time suggesting its renewal by stem cells. In their final set of experiments, they grafted wild-type mouse ovary to transgenic GFP-positive mouse ovarian bursa, and they were able to observe GFP positive oocytes 3-4 weeks later in the grafted ovarian tissue concluding that oogenesis continues in postnatal ovary.

After publication of this landmark paper, a heated debate ignited in the field of reproductive biology followed by studies supporting the presence of oogonial stem cells (Bukovsky *et al.* 2005, Johnson *et al.* 2005, Abban & Johnson 2009, Niikura *et al.* 2009, Zou *et al.* 2009, Pacchiarotti *et al.* 2010, Parte *et al.* 2011, White *et al.* 2012, Zhou *et al.* 2014) while others were refuting the findings of Tilly's group (Bristol-Gould *et al.* 2006, Liu *et al.* 2007, Kerr *et al.* 2012, Zhang *et al*. 2012, Zhang *et al.* 2014, Zarate-Garcia *et al.* 2016). The arguments against the ovarian germline stem cells include several points. First of all, it is emphasized that even if oogonial stem cells exist in the mouse ovary, it does not indicate the presence of neo-oogenesis in human ovary due to biological differences in two different mammalian species. Secondly, the cellular markers used by the researchers to identify oogonial stem cells are not specific to the germline. Therefore, it is criticized that the identified cells could not be accurately classified as germline stem cells. For example, Johnson *et al*. (2004) used MVH, also known as Ddx4, as a germline marker in their studies. However, researchers later on demonstrated MVH/Ddx4 expression in brain and kidney tissues in mice suggesting that MVH/Ddx4 cannot be used as a specific marker for germline cells (Liu *et al.* 2007, Zarate-Garcia *et al.* 2016). Liu *et al.* (2007) also reported a lack of expression of meiotic marker Scp3 in their study. In a recent study, using a transgenic mice model genetically targeting another germline-specific marker, stimulated by retinoic acid gene 8 (Stra8), Wang *et al.* (2017) demonstrated oocyte generation in adult mice after targeted ablation of premeiotic germ cells.

Irrespective of these supporting and against reports, it is acknowledged that the lack of detection of proliferative and meiotic markers in the ovarian tissue samples cannot disprove the existence of the oogonial stem cells as these cells may remain quiescent without showing any significant activity. To investigate whether quiescent oogonial stem cells exist, Zhang *et al*. (2014) specifically ablated growing follicles in a transgenic mice model and examined whether quiescent stem cells would activate to

replenish diminished reserve. Evaluations at 2, 6 and 12 months after follicle ablation showed no oocytes in mice ovaries suggesting the non-existence of quiescent germline stem cells. However, these findings were also objected as the factors which can activate germline stem cells might be different, and depletion of follicle reserve might not be adequate to induce activation.

Another issue with the identification of these stem cells is related to their unknown location such as ovarian cortex, surface epithelium or ovarian medulla. Hence, it is possible that studies focused on certain parts of the ovary might have overlooked these cells to detect. Additionally, in vitro culture conditions for studying oogonial stem cells can be unreliable to represent physiological conditions of the ovarian cellular microenvironment. Besides, culture conditions may fail to replicate hormonal and paracrine signaling conditions that exist in vivo. Therefore, identification and culture of oogonial stem cells possess many challenges for in vitro studies.

Ovarian Stem Cells of Extragonadal Origin

Some researchers alternatively proposed that the stem cells identified in the ovaries may have been originated from other organs and later migrated to the ovary. Lee *et al*. (2007) and Selesniemi *et al*. (2009) reported that bone-marrow derived stem cells migrate to ovaries after bone-marrow transplantation and contribute oogenesis. On the other hand, in a parabiosis model with GFP positive mice, Eggan *et al*. (2006) demonstrated that there was no evidence of circulating cells contributing to ovulated oocytes. Therefore, it is still unclear whether bone-marrow derived stem cells take a role in oogenesis and their primary function in ovarian biology needs to be further investigated. It is a possibility that migrated extragonadal stem cells to the ovary can enhance folliculogenesis by primarily supporting the ovarian microenvironment.

Ovarian Stem Cells and Reproductive Aging

One of the critical questions regarding the existence of ovarian germline stem cells is that if this previously unknown source could replenish follicle reserve, then why women go through menopause? Researchers have proposed different answers to this question. One of the most accepted explanations is that stem cell functions would also decline by aging as seen in male germline leading to loss of renewal potential (Ermolaeva *et al.* 2018). Another possible explanation is that although oogonial stem cells maintain their differentiation into oocytes, the functional deterioration of supporting follicular cells such as granulosa and theca cells may result in loss of follicle reserve. In another theory, researchers proposed that systemic age-related signals may cause ovarian function decline despite the presence of oogonial stem cells (Niikura *et al.* 2009).

Potential Applications of Ovarian Stem Cells

Although the existence of oogonial stem cells is still not conclusively proven, it has the great potential to revolutionize reproductive medicine and transform current treatment options for female infertility and gonadal endocrine insufficiency (Truman *et al.* 2017). Infertility and ovarian failure due to gonadotoxic treatments such as chemotherapy and radiation therapy have become important issues with an increasing rate in the modern age. In the last two decades, various assisted reproductive technologies and fertility preservation methods including ovarian tissue cryopreservation and transplantation procedures have been developed and successfully introduced to the clinical practice (Oktay *et al.* 2017, Taylan & Oktay 2017, Taylan & Oktay 2019). However, these available options also have certain limitations that restrict their application to all patients. Therefore, ovarian germline stem cells hold the potential to provide treatment with a wide range of application in reproduction and endocrine function maintenance.

Conclusion

Based on our current knowledge, there is an accumulating data supporting the potential existence of postnatal oogenesis in the adult mammalian ovary. However, there are still several limitations particularly in the identification of the ovarian germline stem cells which require markers specific to the oogonial stem cells. Another technical limitation is obtaining human ovarian tissue samples preferably from reproductive age women for stem cell research. Although experimental animal studies in mammalian species can demonstrate the presence of oogonial stem cells, this cannot lead to the same conclusion unless these stem cells are clearly shown in the human ovary. Therefore, studies in human ovarian samples have critical value. Despite these limitations, there have been significant advances in molecular biology and reproductive medicine, and these developments will most likely lead us to a conclusive answer in the search of stem cells in the human ovary and may provide novel treatment options for female infertility and menopause-related health conditions.

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Örnek:

Tek yazarlı Makale için

Soyadı, A. Yıl. Makalenin adı. (Sözcüklerin ilk harfi küçük). *Yayınlandığı derginin açık ve tam adı*, Cilt(Sayı)**:** Sayfa aralığı.

Kıvan, M. 1998. *Eurygaster integriceps* Put. (Heteroptera: Scuteleridae)'nin yumurta parazitoiti *Trissolcus semistriatus* Nees (Hymenoptera: Scelionidae)'un biyolojisi üzerinde araştırmalar*. Türkiye Entomoloji Dergisi*, 22(4): 243-257.

İki ya da daha çok yazarlı makale için

Soyadı1, A1. & Soyadı2**,** A2. Yıl. Makalenin adı**.** (Sözcüklerin ilk harfi küçük). *Yayınlandığı derginin tam adı*, Cilt(Sayı): Sayfa aralığı.

Lodos, N. & Önder, F. 1979. Controbution to the study on the Turkish Pentatomoidea (Heteroptera) IV. Family: Acanthasomatidae Stal 1864. *Türkiye Bitki Koruma Dergisi*, 3(3): 139-160.

Soyadı1, A1., Soyadı2, A2. & Soyadı3, A3**.** Yıl. Makalenin adı**.** (Sözcüklerin ilk harfi küçük). *Yayınlandığı derginin tam adı*, Cilt (Sayı): Sayfa aralığı.

Önder, F., Ünal, A. & Ünal, E. 1981. Heteroptera fauna collected by light traps in some districts of Northwestern part of Anatolia. *Türkiye Bitki Koruma Dergisi***,** 5(3): 151-169.

Kitap: Yazarın soyadı, adının baş harfi, basıldığı yıl. Kitabın adı (varsa derleyen veya çeviren ya da editör), cilt numarası, baskı numarası, basımevi, basıldığı şehir,toplam sayfa sayısı.

Örnek:

Soyadı, A., Yıl. *Kitabın adı*. (Sözcüklerin ilk harfi büyük, italik). Basımevi, basıldığı şehir, toplam sayfa sayısı s./pp.

Önder F., Karsavuran, Y., Tezcan, S. & Fent, M. 2006. *Türkiye Heteroptera (Insecta) Kataloğu*. Meta Basım Matbaacılık, İzmir, 164 s.

Lodos, N., Önder, F., Pehlivan, E., Atalay, R., Erkin, E., Karsavuran, Y., Tezcan, S. & Aksoy, S. 1999. Faunistic *Studies on Lygaeidae (Heteroptera) of Western Black Sea, Central Anatolia and Mediterranean Regions of Turkey.* Ege University, İzmir, ix + 58 pp.

Kitapta Bölüm: Yazarın soyadı, adının baş harfi basıldığı yıl. Bölüm adı, sayfa numaraları. Parantez içinde: Kitabın editörü/editörleri, *kitabın adı*, yayınlayan şirket veya kurum, yayınlandığı yer, toplam sayfa sayısı.

Örnek:

Soyadı, A., Yıl. Bölüm adı, sayfa aralığı. In: (editör/editörler). *Kitabın adı*. (Sözcüklerin ilk harfi büyük, italik). Basımevi, basıldığı şehir, toplam sayfa sayısı s./pp.

Jansson, A. 1995. Family Corixidae Leach, 1815—The water boatmen. Pp. 26–56. In: Aukema, B. & Rieger, Ch. (eds) Catalogue of the Heteroptera of the Palaearctic Region. Vol. 1. Enicocephalomorpha, Dipsocoromorpha, Nepomorpha, Gerromorpha and Leptopodomorpha. The Netherlands Entomological Society, Amsterdam, xxvi + 222 pp.

Kongre, Sempozyum: Yazarlar, Yıl. "Bildirinin adı (Sözcüklerin ilk harfi küçük), sayfa aralığı". Kongre/Sempozyum Adı, Tarihi (gün aralığı ve ay), Yayınlayan Kurum, Yayınlanma Yeri.

Örnek:

Bracko, G., Kiran, K., & Karaman, C. 2015. The ant fauna of Greek Thrace. 6th Central European Workshop of Myrmecology, 33-34. July, Debrecen-Hungary.

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Soyadı**,** A. Yıl. Çalışmanın adı. (Sözcüklerin ilk harfi küçük). (web sayfası) **http://www……),** (Erişim tarihi: Mayıs 2009).

Hatch, S., 2001. Studentsperception of online education. Multimedia CBT Systems. http://www.scu.edu.au/schools/sawd/moconf/papers2001/hatch.pdf (Erişim: May 2009).

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