MSC COMMUNICATION IN PHYSIOLOGICAL AND PATHOLOGICAL SETTINGS

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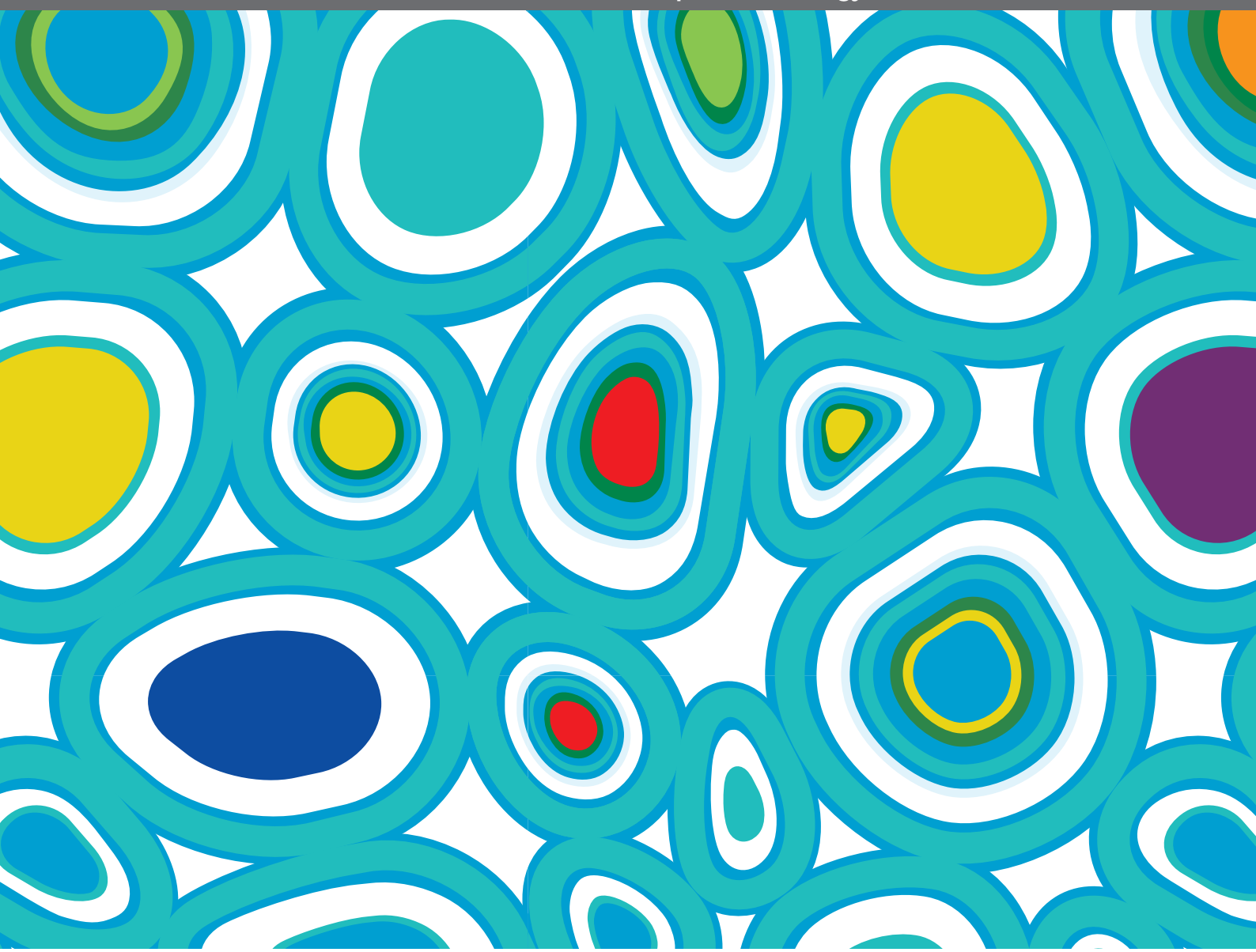

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MSC COMMUNICATION IN PHYSIOLOGICAL AND PATHOLOGICAL SETTINGS

Topic Editors:
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Dr. Bourin works in a biotechnology company that develops a production process of ASC for clinical uses. Dr. Gimble is a co-founder, co-owner, and Chief Scientific Officer at Obatala Sciences and LaCell, biotechnology companies focused on the clinical translation of adipose-derived cells and tissues. The other Guest Editors declare no competing interests with regard to the Research Topic subject.

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EDITORIAL
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Editorial: MSC Communication in Physiological and Pathological Settings

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Editorial on the Research Topic

MSC Communication in Physiological and Pathological Settings

Mesenchymal stromal/stem cells (MSC), first identified within bone marrow, have been studied since the late 1960s. Pioneering studies by Friedenstein and others showed that MSC had the potential for differentiation into osteoblasts, chondrocytes and adipocytes and that, in addition, they supported hematopoiesis. Subsequent work highlighted the presence of similar cells in multiple tissue sites and uncovered other signature MSC properties such as their immunomodulatory and antifibrotic activities. Furthermore, *in vivo* pre-clinical animal models and first-in-human clinical trials have extended these analyses of MSC to evaluate their transplantation actions in many types of disorders. The ability of MSC to communicate with their local microenvironment and systemically is now appreciated as their predominant mechanism of action, making them a privileged tool for regenerative medical applications, through mechanisms of action that are a direct consequence of their microenvironmental and/or systemic communication capacity. However, the means of communication can be varied according to the physiological or pathophysiological situation and may include the secretion of small molecules, cytokines, adipokines, microRNA, and exosome/vesicles as well as direct cell-cell interactions mediated via surface receptors. This special issue contains a series of articles over-viewing the current status of MSC's communication avenues in the context of pathophysiological settings and clinical translation. These nine papers focus on two central themes (Planat-Benard et al., 2021): the crosstalk of MSC and immune cells and; (2) the involvement of MSC in tissue physiology and pathophysiology.

The first theme, immune modulation by MSC, is an emerging topic of discovery. Bazzoni et al. review the crosstalk of MSC with the immune cells via extracellular vesicles. They evaluate pleiotropic action of MSC relevant to the pathways involving both for innate and adaptative immune systems. Stevens et al. extend this theme with an in depth look at the crosstalk between MSC and the mononuclear phagocytic system. This manuscript presents multimodal data relating to paracrine secretion, metabolic reprogramming, organelle donation, extracellular vesicles and contact dependent communications. Planat et al., emphasizes that a common and central point between the MSC features of multipotency paracrine activity, and physical cell-cell interactions, especially with immune inflammatory cells, is the key importance of metabolism that governs their fate and behaviors. Finally, Kang et al. present experimental data showing that macrophages exert considerable influence on the differentiation of MSC in pathophysiological conditions. They report that in type 2 diabetes mellitus, macrophages at rest display an inflammatory phenotype and impaired bone regeneration.

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Manuscripts within the second theme explore the role of MSC in various physiological or pathological conditions. Two manuscripts examine the interactions between the MSC and the other cells of the bone microenvironment. Arthur and Gronthos review the role of Eph-Ephrin system on bone physiology and hematopoiesis, showing that multiple Eph receptors and Ephrin ligands underpin MSC physiology, cartilage and bone homeostasis or hematopoiesis. In a related study, Takam Kamga et al. bring an interesting perspective to the role of the Notch and Wnt pathways as they relate to communication between MSC and hematopoietic cells in both healthy and leukemic subjects. They demonstrate how physiological signaling can be hijacked by cancer cells for their own protection at the expense of the neighboring MSC. Two experimental manuscripts provide support for a potential role of MSC to mitigate pathological conditions. Chu et al. show that amnion MSC can reduce the impact of hypoxia on trophoblasts. This effect is correlated with downmodulation of the mTOR pathway via EZH2 and hypothetically could be the mechanism of support for a clinical trial on the use of MSC therapy to mitigate or treat preeclampsia. Lu et al. explore the benefit of MSC conditioned medium to protect endothelium via a multifactorial mechanism involving both protein growth factors and small molecules such as carbon monoxide. Chiabotto et al. critically review the preclinical data showing that MSC impact liver fibrosis via mechanisms involving MSC engraftment and differentiation along the hepatic lineage as well as secretion of extracellular vesicles exerting anti-inflammatory and anti-fibrotic effects.

Together, this body of work provides a topical summary of the current status of our understanding of MSC communication in health and disease. We hope that these contributions will provide both insights and inspiration to the next generation of investigators exploring the fields of stromal/stem cell biology and regenerative medicine.

AUTHOR CONTRIBUTIONS

PB wrote the manuscript, JMG, LC, and AS corrected it. All approved the final manuscript.

REFERENCE

Planat-Benard, V., Varin, A., and Casteilla, L. (2021). MSCs and Inflammatory Cells Crosstalk in Regenerative Medicine: Concerted Actions for Optimized Resolution Driven by Energy Metabolism. *Front. Immunol.* 12, 626755. doi:10.3389/fimmu.2021.626755

Conflict of Interest: Author JMG is a co-founder, co-owner, and employee of the companies LaCell LLC and Obatala Sciences, for profit biotech companies focusing on adipose derived stromal/stem cell clinical translation. Author LC is a shareholder of cell-easy and have a consultancy activity for this company.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
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Distinct Factors Secreted by Adipose Stromal Cells Protect the Endothelium From Barrier Dysfunction and Apoptosis

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We have shown previously that adipose stromal cell (ASC)-derived conditioned media (CM) limited lung injury, endothelial barrier dysfunction, and apoptosis. Here, we used endothelial hyperpermeability and apoptosis assays to investigate how concentration processes affect endothelium-directed bioactivity of ASC-CM and to gain information on the nature of bioactive factors. Comparison of ASC-CM concentrated with differential molecular weight (MW) cutoff filters showed that endothelial barrier protection depended on the species-specific factors in ASC-CM fractionated with MW $>$ 50 kDa. Known barrier regulators—keratin growth factor (KGF), vascular endothelial growth factor (VEGF), and hepatocyte growth factor (HGF)—were detected in ASC-CM fraction of $>$ 100 kDa. Pretreatment of endothelial monolayers with concentrations of KGF, VEGF, and HGF detected in ASC-CM showed that only KGF and HGF protect the endothelium from barrier dysfunction. Depletion of KGF and HGF from ASC-CM attenuated ASC-CM's ability to protect the endothelial barrier. In contrast to barrier-protective factors, apoptosis-protective factors fractionated with MW $<$ 3 kDa and were not species-specific. Application of donors of apoptosis-mitigating gases showed that the CO donor carbon monoxide-releasing molecule 2 (CORM2) protected the endothelium from apoptosis, while the H_2S donor NaSH did not. Knockdown of CO-generating heme oxygenase 1 in ASC attenuated ASC-CM's ability to protect the endothelium from apoptosis. We have shown that tumor necrosis factor alpha (TNFα)-induced apoptosis in endothelium is c-Jun N-terminal kinase (JNK)-dependent, and JNK activation is inhibited by ASC-CM pretreatment of endothelial cells. ASC-CM from heme oxygenase 1-depleted ASC displayed attenuated ability to suppress endothelial JNK activation, suggesting that CO-mediated protection of the endothelium from apoptosis is achieved by the downregulation of the JNK pathway. Altogether, our results demonstrate that the concentration of ASC-CM with low MW cutoff filters significantly reduces its anti-apoptotic activity while preserving its barrier-protective activity.

Keywords: ASC, conditioned media, endothelial cells, barrier dysfunction, apoptosis

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INTRODUCTION

Adipose stromal cells (ASC) are a population of adult mesenchymal stromal (stem) cells first isolated from adipose tissue in Zuk et al. (2001). They have self-renewing properties and can differentiate into several cell lineages, but like other stromal cells, their therapeutic potential is thought to be associated with the secretion of protective and regenerative factors rather than engraftment and trans-differentiation (Liang et al., 2014). The therapeutic potential of ASC has been shown in several models of disease (Feisst et al., 2015). On the basis of these experimental studies, ASC entered clinical trials for diverse indications including musculoskeletal disorders, fistula, ulcers following peripheral artery disease, multiple sclerosis, myocardial infarction and stroke, COPD, and pulmonary fibrosis (can be reviewed at https://stemcellsportal.com/ifats-clinical-trials-view). Significant promise shown by ASC (Bateman et al., 2018) led to therapy progression to phase II and phase III clinical trials and regulatory approval for Crohn's fistula (Galipeau and Sensebe, 2018; Olsen et al., 2018).

Since anti-inflammatory, anti-apoptotic, proliferative, angiogenic, immunomodulatory, and antioxidant effects displayed by stromal cells are attributed to secreted factors (Liang et al., 2014), cell-free stromal cell secretome can represent an alternative therapy for the treatment of pathologic conditions benefiting from stromal cell therapy. Secretome preparations can be standardized, generated and distributed in advance, and will not require specialized equipment/facilities currently needed for the isolation of ASC-containing stromal vascular fraction cells or storage of expanded stromal cells. In preclinical studies, we and others have demonstrated that ASC-conditioned media (ASC-CM) effectively suppresses a variety of pathologies ranging from bone loss (Li et al., 2018) and neurodegeneration (Fontanilla et al., 2015) to kidney (Bi et al., 2007) and lung injury (Lu et al., 2015). Methods of CM preparation for preclinical and early clinical studies were primarily tailored to the intended mode of administration while being influenced by somewhat fragmentary knowledge about the distribution of biological activity among various fractions. Unconcentrated CM, CM concentrates with low molecular weight (MW) cutoffs (Pawitan, 2014), and extracellular vesicle/exosome preparations (Giebel et al., 2017) were found to be effective in different preclinical and clinical studies. However, the future development of CM preparation as a therapeutic will rely on the ability to optimize the manufacturing process to balance maximizing bioactivity with the cost-efficient manufacturing, distribution, and storage of the final product. Whereas broad preclinical effects of CM preparations speak to their wide-ranging and robust therapeutic potential, the multifactorial nature of CM presents the challenge of product standardization, which necessitates elucidation of indication-relevant bioactive components in CM and the development of indication-relevant bioactivity tests.

We and others have recently shown that stromal cell CM effectively suppresses indices of lung injury *in vivo* (Ionescu et al., 2012; Lu et al., 2015). Lung injury has a complex pathology involving excessive inflammatory response, concomitant with the loss of endothelial and epithelial barrier function and subsequent lung cell death (Thompson et al., 2017). In the present study, we used endothelial responses relevant to lung injury, namely barrier dysfunction and apoptosis, to analyze partitioning of ASC-CM biological activities based on MW. Here, we show that the protection from apoptosis and barrier dysfunction is rendered by two non-overlapping fractions of ASC-CM. We found that barrier-protective properties of ASC-CM are preserved in CM subjected to concentration with low MW cutoff filters, while apoptosis-protective properties are significantly reduced by the concentration process.

MATERIALS AND METHODS

Materials

Forty-kDa fluorescein isothiocyanate (FITC)-dextran, NaSH, and antibody to β-actin (1/10,000 dilution used) were purchased from Sigma (St. Louis, MO). Antibodies to cleaved caspase 3, phospho- and pan-p38, phospho- and pan-ERK, and phospho- and pan-c-Jun N-terminal kinase (pan-JNK) were from Cell Signaling (Beverly, MA) and were used at 1/1,000 dilution. CD81 and CD63 antibodies (1/1,000 dilution) and heme oxygenase 1 siRNA were from Santa Cruz Biotechnology (Dallas, TX). Heme oxygenase 1 antibody (1/1,000) and hepatocyte growth factor (HGF) siRNA were from Thermo Fisher Scientific (Waltham, MA). HRP-conjugated anti-rabbit and anti-mouse antibodies were from Cell Signaling and were used at 1/3,000. JNK inhibitor II and 3, 10, 50, and 100 kDa cutoff centrifugation filter inserts were from EMD Millipore (Billerica, MA). Carbon monoxide-releasing molecule 2 (CORM2) was from Tocris Bioscience (Minneapolis, MN).

Cell Culture

All procedures for collecting human adipose tissue were approved by the Indiana University School of Medicine Institutional Review Board. Human and rat ASC were isolated from subcutaneous adipose tissue samples and characterized as described in Lu et al. (2015). Human pulmonary artery endothelial cells (HPAEC) were purchased from Lonza (Walkerville, MD) and used at passages 5–8. Rat lung microvascular endothelial cells (RLEC) were kindly provided by Dr. Irina Petrache (Schweitzer et al., 2011) (Indiana University) and used up to passage 16, at which transendothelial electrical resistance (TER) levels characteristic of endothelial monolayers were consistently observed. RLEC were maintained in DMEM-high glucose supplemented with 10% FBS and 1% penicillin–streptomycin. Both ASC and HPAEC were propagated using endothelial growth media 2-microvascular (EGM2-MV) (Lonza).

ASC-CM Generation

Conditioned media from rat and human ASC were generated by incubating subconfluent ASC (250,000 cells/ml) with EGM-2MV for 48 h. To deplete ASC-CM of specific factors, 50 nM siRNA mixed with DharmaFECT 1 (Dharmacon, Lafayette, CO) was applied to 30% confluent ASC for 24 h.

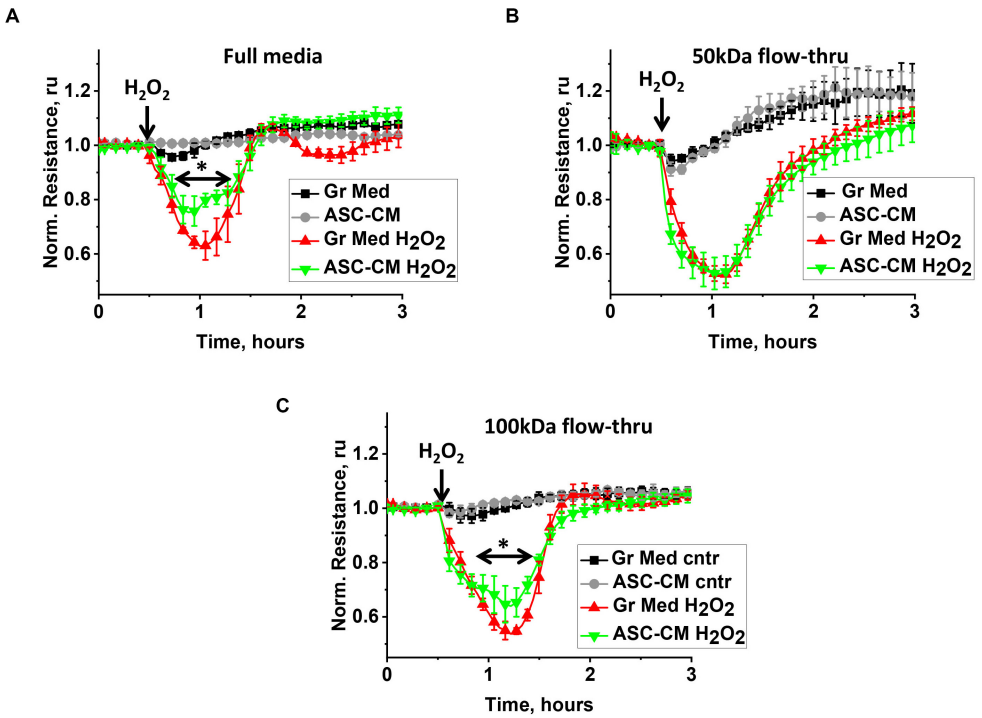

FIGURE 1 | ASC-CM factors preserving transendothelial permeability partition with MW > 50 kDa. HPAEC grown on gold electrodes of ECIS arrays were exposed to **(A)** the original growth media (black, red) or ASC-CM (gray, green), **(B)** 50 kDa flow-through fractions of the growth media (black, red) or of ASC-CM (gray, green), and **(C)** 100 kDa flow-through fractions of the growth media (black, red) or of ASC-CM (gray, green). Media were removed after 72 h of pretreatment, and HPAEC were challenged with 250 μM H_2O_2 (red, green) or vehicle (black, gray). Data are presented as mean ± SEM from three parallel recordings; the results were reproduced in at least three independent experiments. *Repeated measurement one-way ANOVA detected significant differences in **(A)** responses of HPAEC exposed to unmanipulated ASC-CM (green) when compared to unmanipulated growth media (red) and **(C)** responses of HPAEC exposed to 100 kDa flow-through of ASC-CM (green) when compared to 100 kDa flow-through of growth media (red).

ASC-CM Manipulation

To heat-inactivate media, batches of ASC-CM and EGM-2MV were subjected to 30 min in boiling water bath, followed by 10 min centrifugation at 10,000 × *g* and collection of the supernatant. To deplete exosomes, batches of ASC-CM and EGM-2MV were subjected to 70 min ultracentrifugation at 100,000 × *g* using Sorvall ultracentrifuge, and the supernatant was collected and used for analyses. The same batches of heat-inactivated and exosome-depleted media were used to perform barrier dysfunction and apoptosis assays. For Western blot analysis, pellet from ultracentrifugation was dissolved in 1% sodium dodecyl sulfate (SDS) on PBS and analyzed with CD81 and CD63 antibodies. To fractionate media, ASC-CM and EGM-2MV were concentrated by centrifugation at 4,000 × *g* using 3, 10, 50, and 100 kDa filter inserts; 0.4 mg/ml solution of 40 kDa FITC-dextran was subjected to similar fractionation to verify the fractionation method. Fractionation was stopped when 40% of the initial volume remained.

ELISA Analyses

Quantikine ELISA kits for human HGF and KGF were from R&D Systems (Minneapolis, MN). Vascular endothelial growth factor (VEGF) content was determined using sandwich ELISA with anti-human VEGF capturing and biotinylated detecting antibody and streptavidin-horse radish peroxidase complex (R&D Systems). ASC-CM was concentrated with 3 kDa filter (KGF) or diluted (HGF, VEGF) to allow detection in the linear range.

Measurement of Transendothelial Permeability

TER was measured using Electrical Cell-Substrate Impedance Sensing (ECIS) (Applied Biophysics, Troy, NY) as described previously (Bogatcheva et al., 2009). HPAEC or RLEC plated on gold electrodes of ECIS array chambers were exposed to 1:1 mixture of EGM-2MV and test media for 48–72 h. In growth factor supplementation experiments, endothelial cells were exposed to the indicated concentration of growth factors for 48–72 h. At the end of the pre-incubation period, endothelial resistance reached 1,200–1,400 Ω for HPAEC and 1,800–2,000 Ω for RLEC, evident of monolayer confluence. Media were changed to basal media EBM-2 (Lonza) 2 h prior to the beginning of TER recording.

Western Immunoblotting

HPAEC or RLEC grown in 12-well plates were exposed to 1:1 mixture of EGM-2MV and test media for 72 h; media was changed to EBM-2 1 h prior to the beginning of stimulation. Cells

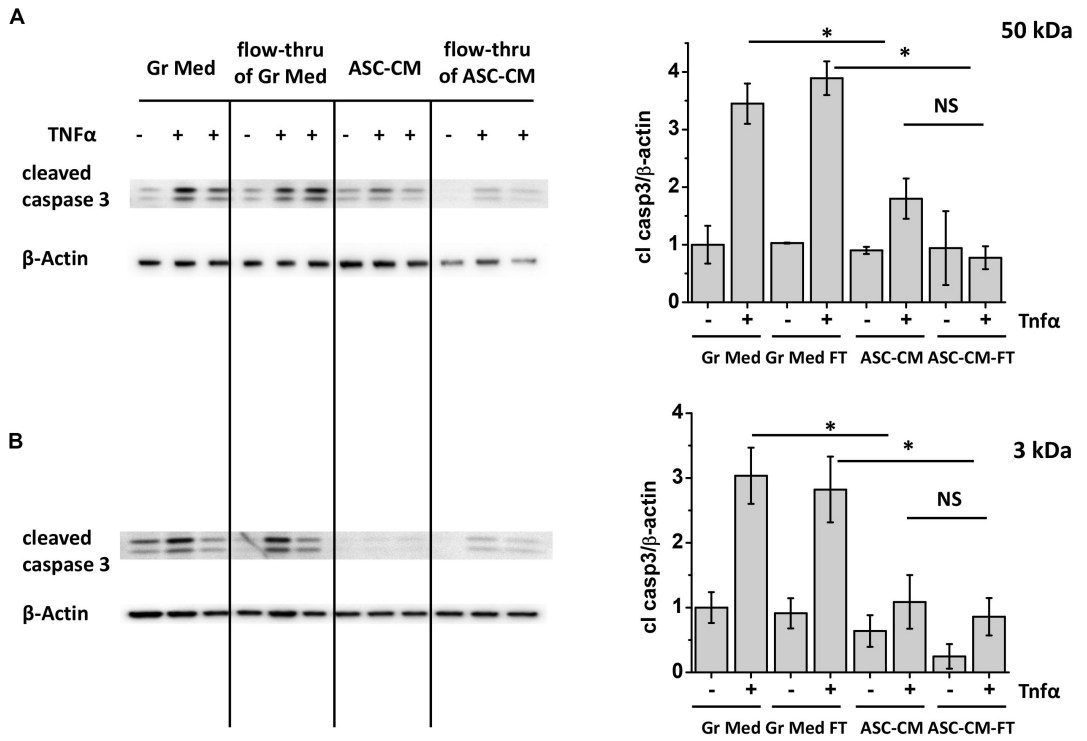

FIGURE 2 | ASC-CM factors preventing endothelial apoptosis partition with MW < 3 kDa. HPAEC pretreated with the original growth media and ASC-CM, or the 50- **(A)** or 3-kDa **(B)** flow-through fractions of growth media and ASC-CM were challenged with 2 ng/ml TNFα for 4 h. Cell lysates were analyzed with antibodies to cleaved caspase 3 and β-actin (loading control). Data from three independent experiments were pooled and presented as cleaved caspase 3/β-actin ratio mean ± SEM. One-way ANOVA with Tukey *post hoc* was used to detect whether differences between indicated columns are significant *($p < 0.05$) or not significant (NS).

were stimulated with 2 ng/ml tumor necrosis factor (TNF) for the times indicated in the figure legends. ASC grown in 12-well plates were treated with siRNA as described in the figure legends. Protein extracts were prepared by lysing cells with 1% SDS-containing buffer and separated on 4–20% polyacrylamide gels followed by transfer to nitrocellulose membrane. After staining with specific antibodies, a signal was developed, imaged, and quantified with Bio-Rad imaging system.

Statistical Analysis

Repeated measures one-way ANOVA (GraphPad Prism 6) or one-way ANOVA with Tukey *post hoc* was used to analyze TER recordings. One-way ANOVA with Tukey *post hoc* or *t*-test with Welch's correction (unequal variance) was used to analyze Western blot and ELISA results. A probability value of < 0.05 was considered statistically significant.

RESULTS

Endothelial-Protective Factors in ASC-CM Are Detected in > 50 kDa Fraction, Whereas Apoptosis-Protective Factors Are Detected in < 3 kDa Fraction

We had previously shown that preconditioning of the endothelium with ASC-secreted factors protects it from hyperpermeability and activation of pro-apoptotic pathways; a protective effect was not detected when the endothelium was preconditioned with dermal fibroblast-secreted factors (Lu et al., 2015). To understand how the biological activity of ASC-CM is partitioned among different MW fractions, we compared the abilities of the original CM and its various flow-through fractions to attenuate H_2O_2-induced endothelial barrier dysfunction and TNFα-induced endothelial apoptosis. **Figure 1A** shows the typical response of the control endothelium to H_2O_2 stimulation. H_2O_2 challenge of monolayers pretreated with control media led to a dramatic decrease of TER within the first 15–30 min, followed by the period of barrier restoration. HPAEC pretreated with unmanipulated ASC-CM also manifested a decrease in TER, however, restoration of barrier function occurred faster (**Figure 1A**), minimizing the duration and severity of endothelial barrier leakage. Exposure of the endothelium to the ASC-CM flow-through fractions obtained with MW cutoffs of 50 kDa (**Figure 1B**) or less (not shown) did not result in endothelial protection from the barrier-disruptive effect of H_2O_2. Only the 100-kDa flow-through fraction of ASC-CM manifested a barrier-protective activity (**Figure 1C**).

Figure 2A shows the typical response of control media-pretreated HPAEC to TNFα stimulation. Challenge of control monolayers with TNFα led to a marked increase in caspase 3 cleavage, indicative of pro-apoptotic activation of endothelium. This process was attenuated in monolayers pretreated with unmanipulated ASC-CM. Exposure of the endothelium to the

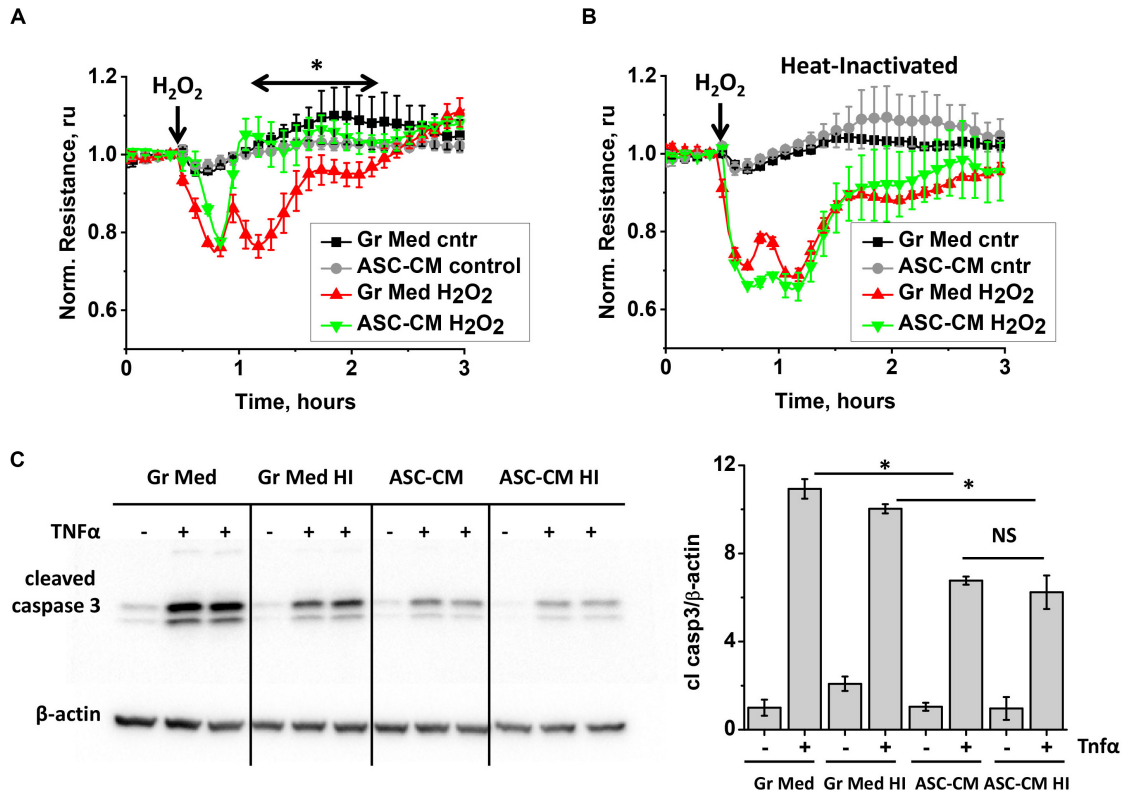

FIGURE 3 | ASC-CM factors preserving transendothelial permeability are heat-sensitive, whereas factors preventing endothelial apoptosis are not. **(A,B)** HPAEC grown on gold electrodes of ECIS arrays were exposed to **(A)** the original growth media (black, red) or ASC-CM (gray, green) and **(B)** heat-inactivated growth media (black, red) or ASC-CM (gray, green). After 72 h of preconditioning, media were substituted with EBM-2, and HPAEC were challenged with 250 μM H_2O_2 (red, green) or vehicle (black, gray). Shown are the means ± SEM of three parallel recordings; the results were reproduced in at least three independent experiments. **(C)** HPAEC pretreated with the original or heat-inactivated (HI) growth media and ASC-CM were challenged with 2 ng/ml TNFα for 4 h. Cell lysates were analyzed with antibodies to cleaved caspase 3 and β-actin (loading control). Data from three independent experiments were pooled and presented as cleaved caspase 3/β-actin ratio mean ± SEM. One-way ANOVA with Tukey *post hoc* was used to detect whether differences between indicated columns are significant *($p < 0.05$) or not significant (NS).

ASC-CM flow-through fractions obtained with MW cutoffs of 50 kDa resulted in significant endothelial protection from the pro-apoptotic induction by TNFα (**Figure 2A**). Moreover, 10 (not shown) and 3 kDa flow-through fractions were similarly effective in the prevention of pro-apoptotic response to TNFα (**Figure 2B**).

Heat Inactivation and Depletion of Extracellular Vesicles From ASC-CM Affects Endothelial Barrier Protection but Not Apoptosis Protection

To gain more information about the nature of bioactive components in ASC-CM, we subjected ASC media to several manipulations. First, we heat-inactivated ASC–CM and assessed its ability to confer endothelial protection from barrier dysfunction and apoptosis. Comparison of responses of HPAEC monolayers pretreated with unmanipulated (**Figure 3A**) and heat-inactivated (**Figure 3B**) ASC-CM shows that heat inactivation results in a loss of ASC-CM's ability to protect the endothelium from barrier dysfunction.

Remarkably, heat inactivation of ASC-CM did not void its ability to protect the endothelium from apoptosis (**Figure 3C**), suggestive of the heat-stable nature of apoptosis-protective factors.

To further characterize the active components in ASC-CM, we subjected ASC-CM to ultracentrifugation at 100,000 × *g* to deplete extracellular vesicles (EV), which have been shown to mediate many beneficial activities of stromal cells (Fujita et al., 2018). Exposure of the endothelium to the control growth media subjected to ultracentrifugation did not affect its response to H_2O_2 (data not shown). However, exposure of the endothelium to EV-depleted ASC-CM revealed that its barrier-protective potency is significantly, but not completely, attenuated as a result of ultracentrifugation (**Figure 4A**). To ensure that the duration of ultracentrifugation was sufficient to remove EV from ASC-CM, we subjected the supernatant from the first round of ultracentrifugation to a second, consecutive round. The pellets from both rounds were collected and analyzed for the presence of exosomal markers CD81 and CD63 (tetraspanin). CD81 and CD63 staining was positive in the pellet resultant from the first, but not the second ultracentrifugation (**Figure 4B**), suggesting

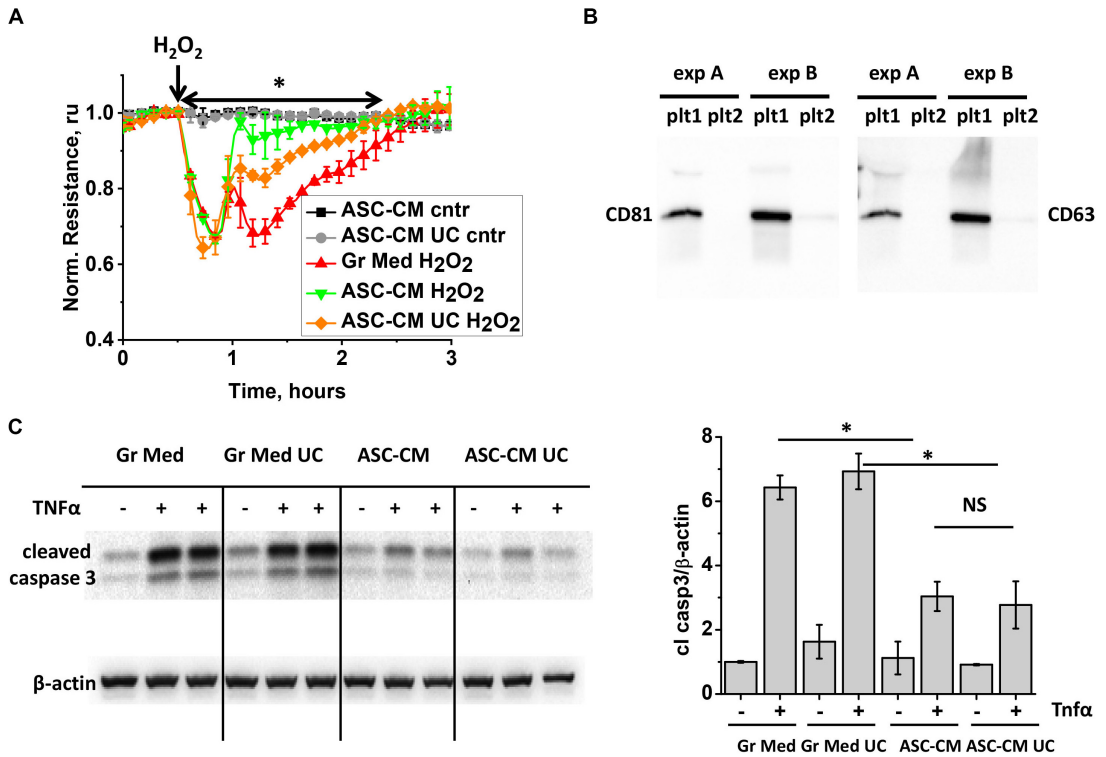

FIGURE 4 | ASC-CM factors preserving transendothelial permeability are partially associated with extracellular vehicles, whereas factors preventing endothelial apoptosis are not. **(A)** HPAEC grown on gold electrodes of ECIS arrays were exposed to the original growth media (black, red), ASC-CM (green), or EV-depleted supernatant of ASC-CM subjected to 100,000 × *g* ultracentrifugation (gray, orange). After 72 h of preconditioning, media were substituted with EBM-2, and HPAEC were challenged with 250 μM H_2O_2 (red, green, orange) or vehicle (black, gray). Shown are the means ± SEM of three parallel recordings; the results were reproduced in at least three independent experiments. *Repeated measurement one-way ANOVA detected significant differences between responses of HPAEC exposed to the original ASC-CM (green) and ASC-CM subjected to ultracentrifugation (orange). **(B)** ASC-CM were subjected to 10,000 × *g* centrifugation to remove the cell debris, followed by 100,000 × *g* ultracentrifugation, and a subsequent round of 100,000 × *g* ultracentrifugation to remove EV. Pellets from the first (plt1) and second (plt2) rounds of 100,000 × *g* ultracentrifugation were analyzed by Western blotting with CD81 and CD63 antibodies. Results from two independent experiments **(A,B)** are presented. **(C)** HPAEC pretreated with the original and ultracentrifuged (UC) growth media and ASC-CM were challenged with 2 ng/ml TNFα for 4 h. Cell lysates were analyzed with antibodies to cleaved caspase 3 and β-actin (loading control). Data from three independent experiments were pooled and presented as cleaved caspase 3/β-actin ratio mean ± SEM. One-way ANOVA with Tukey *post hoc* was used to detect whether differences between indicated columns are significant *($p < 0.05$) or not significant (NS).

near complete depletion of EV from ASC-CM with one round of ultracentrifugation.

When pro-apoptotic responses of HPAEC were assessed in monolayers pretreated with ASC-CM subjected to ultracentrifugation, we found that EV depletion did not decrease ASC-CM's ability to prevent activation of caspase 3 cleavage (**Figure 4C**).

Endothelial-Protective Factors in ASC-CM Are Species-Specific, While Apoptosis-Protective Factors Are Not

To gain further information about the nature of bioactive factors in ASC-CM, we assessed whether barrier protection and apoptosis protection are exerted in a species-specific manner. First, human endothelial cells were pretreated with CM from rat and human ASC and analyzed for H_2O_2-induced hyperpermeability. Unlike CM from same-species ASC, rat ASC-CM did not confer barrier protection to human endothelium (**Figure 5A**). Next, rat endothelial cells were pretreated with human and rat ASC-CM. While rat ASC-CM exposure attenuated RLEC response to H_2O_2, human ASC-CM did not (**Figure 5B**).

When a similar experiment was conducted to assess the apoptosis-protective properties of xenogeneic ASC-CM, rat ASC-CM conferred protection from TNFα-induced apoptosis in human endothelium (**Figure 5C**).

Barrier-Protective Properties of ASC-CM Can Be Partially Attributed to the Secreted KGF and HGF

We next focused our attention on three factors which are known at certain concentrations to improve endothelial barriers: VEGF (Mirzapoiazova et al., 2006), KGF (Gillis et al., 1999), and HGF (Liu F. et al., 2002). To understand in which fraction of ASC-CM these factors would partition, we assessed the levels of KGF, HGF, and VEGF A in 30, 50, and 100 kDa flow-through fractions.

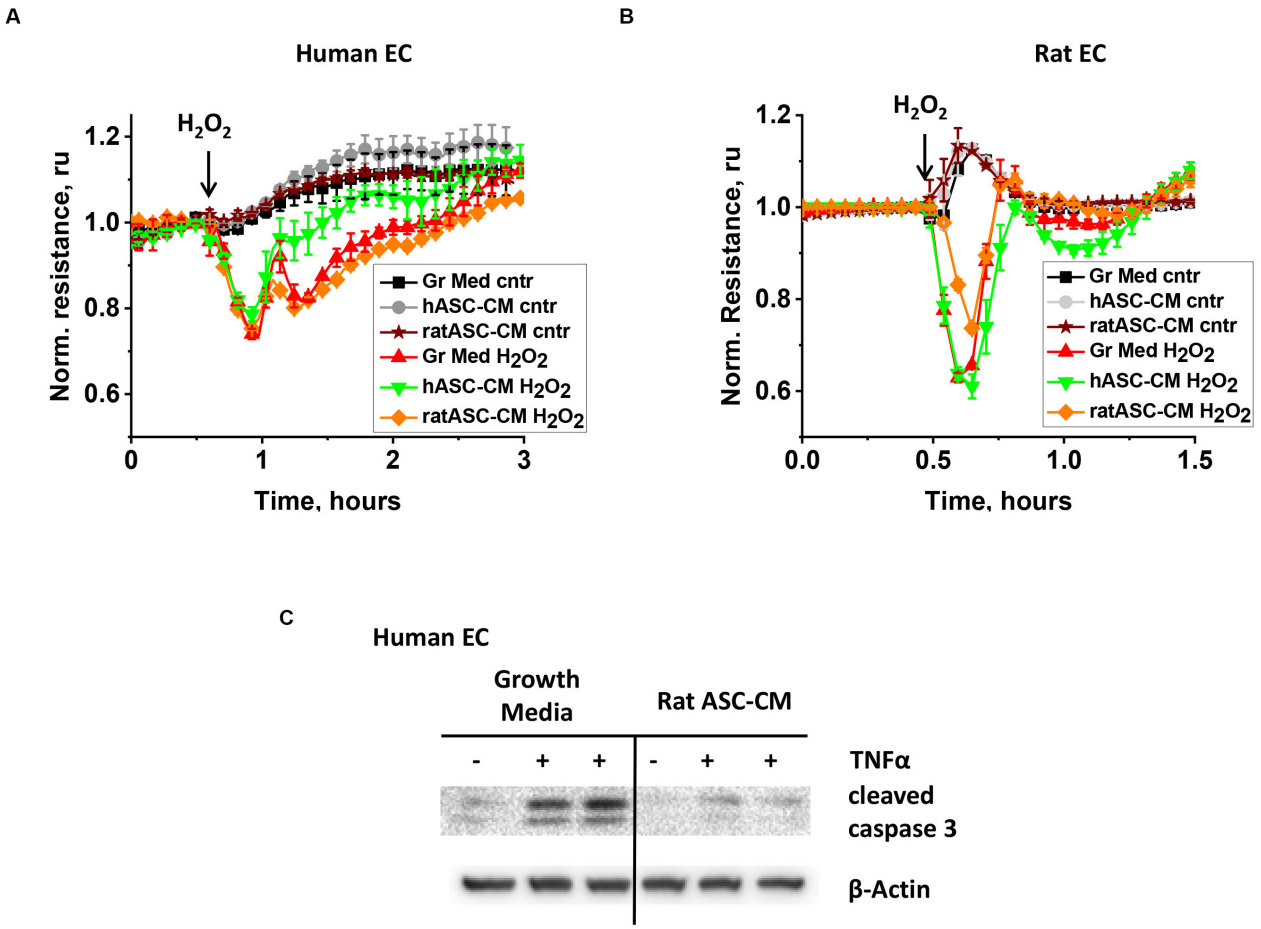

FIGURE 5 | ASC-CM factors protecting from barrier dysfunction are species-specific, whereas anti-apoptotic factors are not. HPAEC **(A)** or RLEC **(B)** grown on gold electrodes of ECIS arrays were exposed to the original growth media (black, red), human ASC-CM (gray, green), or rat ASC-CM (brown, orange). Media were removed after 72 h of endothelial cell exposure, and HPAEC/RLEC were challenged with 250 μM H_2O_2 (red, green, orange) or vehicle (black, gray, brown). Shown are the means ± SEM of three parallel recordings. **(C)** HPAEC were pretreated with growth media or rat ASC-CM. After 72 h, media was substituted with EBM-2; HPAEC were challenged with 2 ng/ml TNFα for 4 h. Cell lysates were analyzed with antibodies to cleaved caspase 3 and β-actin (loading control).

Surprisingly, detectable amounts of KGF (MW ∼19 kDa) and VEGF 121 and VEGF 165 (estimated MW ∼20 kDa, but may migrate as 30 kDa due to glycosylation) appeared in 100 kDa flow-through only (**Figures 6A,C**). HGF (estimated MW for α- and β-chains are 54 and 26 kDa, respectively) also partitioned with higher MW than expected, with only minor fraction detected in 100 kDa flow-through (**Figure 6B**). To ascertain that fractionation *via* 30 and 50 kDa cutoff filters occurred according to specified MW, we subjected 40 kDa FITC-dextran to the filtration. As expected from the product with the normal distribution of MW peaking at 40 kDa, passage of some fluorescent material was detected in 30 kDa flow-through; fluorescence level was higher in 50 kDa flow-through (**Figure 6D**). Altogether, these data show that KGF, VEGF, and HGF fractionate as bigger MW complexes. Importantly, analysis of flow-through rendering barrier protection (**Figures 1A–C**) indicated that 50 kDa and less flow-through fractions deficient in KGF, VEGF, and HGF do not possess barrier-protective properties.

We next proceeded to test whether levels of KGF, VEGF, and HGF detected in ASC-CM would be sufficient to confer barrier protection exerted by ASC-CM. As data of literature suggest that some of the factors, such as KGF, can be associated with EV while secreted by stromal cells (Zhu et al., 2014), we first assessed whether ELISA assays employed to assess factor levels in ASC detect soluble or total levels of factors. For that, we compared the levels of VEGF, KGF, and HGF in the original ASC-CM and ASC-CM depleted by ultracentrifugation. We did not observe decreases in VEGF, HGF, and KGF levels after ultracentrifugation (data not shown), suggesting that the levels indicated in **Figure 6** reflect levels of soluble factors only and not levels of factors associated with EVs. We next supplemented EGM-2MV with the concentrations of soluble VEGF, HGF, and KGF found in ASC-CM (1 ng/ml, 30 ng/ml, and 10 pg/ml, respectively) and pretreated the endothelium for 72 h. Our data indicated that preconditioning of HPAEC with VEGF did not protect the endothelium from H_2O_2-induced barrier disruption (**Figure 7A**). However, exposure of endothelium to KGF and HGF significantly

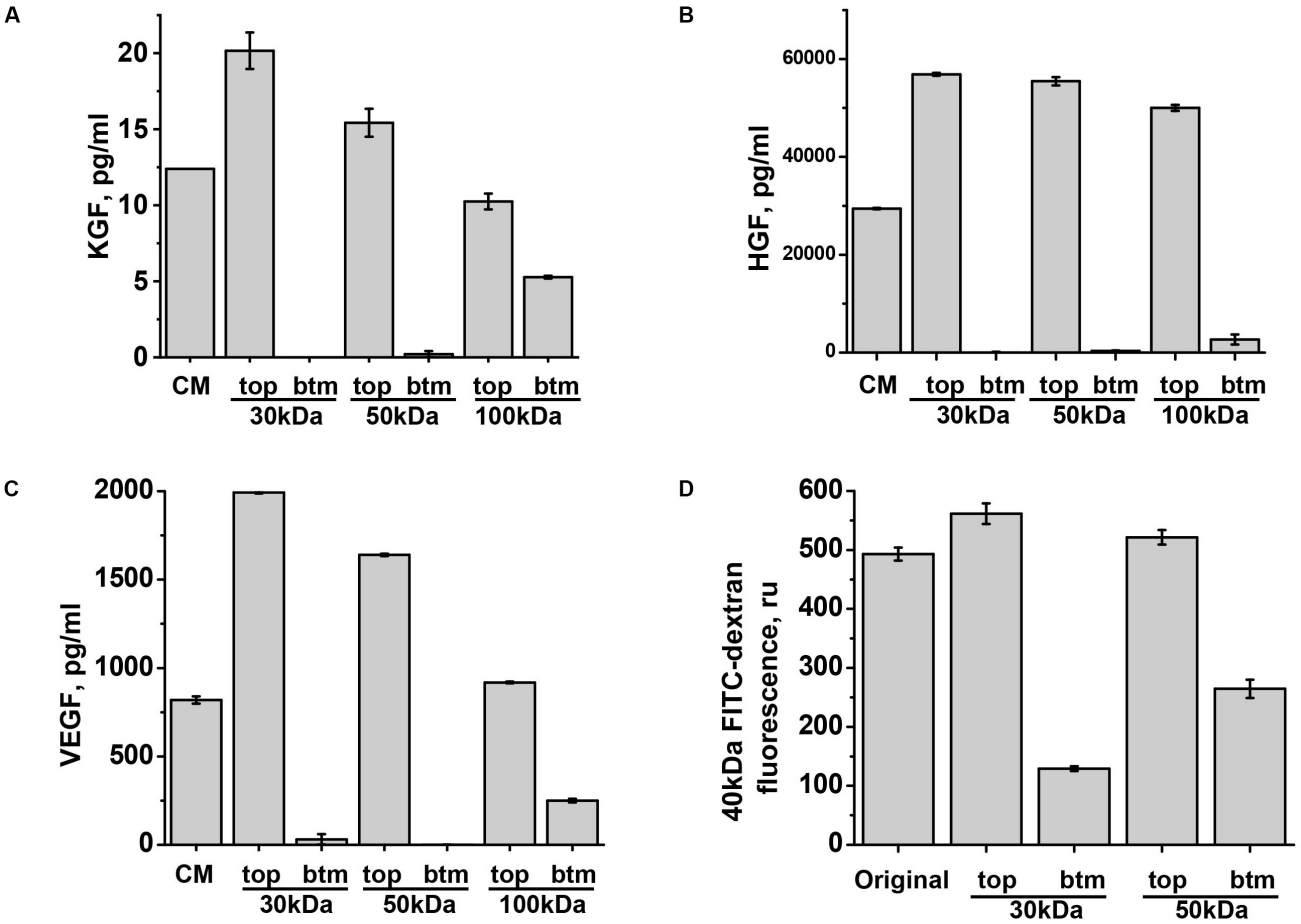

FIGURE 6 | KGF, HGF, and VEGF partition in the fraction with MW exceeding 50 kDa. **(A–C)** ASC-CM was generated over the period of 48 h. KGF, HGF, and VEGF concentrations were determined in the original ASC-CM (CM), and top (tp) and bottom (bt) fractions generated by filtration through indicated MW cutoff membranes. Data from three independent experiments are presented. **(D)** 40 kDa FITC-dextran was filtered *via* indicated MW cutoff membranes; fluorescence levels of the original solution and top and bottom fractions are presented.

suppressed H_2O_2-induced decrease in TER (**Figure 7B**). In concert with these data, ASC with HGF siRNA knockdown yielded ASC-CM with attenuated barrier-protective properties (**Figure 7C**). Similar to HGF depletion, KGF neutralization with anti-KGF antibody attenuated the barrier-protective properties of ASC-CM (**Figure 7D**).

Apoptosis-Protective Properties of ASC-CM Can Be Partially Attributed to the Generated Carbon Monoxide

Since our data indicated that apoptosis protection by ASC-CM is conveyed by heat-stable, non-species-specific factors of low MW, we next assessed the ability of two stable gases with known anti-apoptotic properties, namely carbon monoxide (Liu X.M. et al., 2002; Almeida et al., 2012; Li et al., 2012) and hydrogen sulfide (Sivarajah et al., 2009; Wu et al., 2015), to protect HPAEC from TNFα-induced apoptosis. CO- and H_2S-generating enzymes are known to be expressed in mesenchymal stromal cells (Zarjou et al., 2011; Gambari et al., 2017). To assess whether CO and H_2S can mimic the beneficial effect of ASC-CM, we preconditioned HPAEC with CO donor CORM2 (Babu et al., 2017) and H_2S donor NaHS (Wu et al., 2015). **Figure 8A** shows that CORM2 exposure, but not NaHS exposure, significantly attenuated endothelial caspase 3 cleavage in response to TNFα .

To generate ASC-CM with decreased amount of carbon monoxide, we knocked down CO-generating enzyme heme oxygenase 1 (HO-1) with specific siRNA. We first assessed whether the suppression of HO-1 will be stable after the removal of siRNA for the period required to generate ASC-CM. **Figure 9A** shows that ASC demonstrated sustained changes in HO-1 expression 24 h after the removal of siRNA. These conditions were applied to generate ASC-CM free of HO-1 siRNA, which could have had direct effects on endothelial monolayers.

To assess the effect of CO depletion on ASC-CM apoptosis-protective properties, we preconditioned HPAEC with CM from ASC subjected to HO-1 knockdown. Control ASC-CM pretreatment led to the significant suppression of caspase 3 activation in HPAEC (**Figure 9B**); CM from ASC treated with HO-1 siRNA displayed attenuated potency. Altogether,

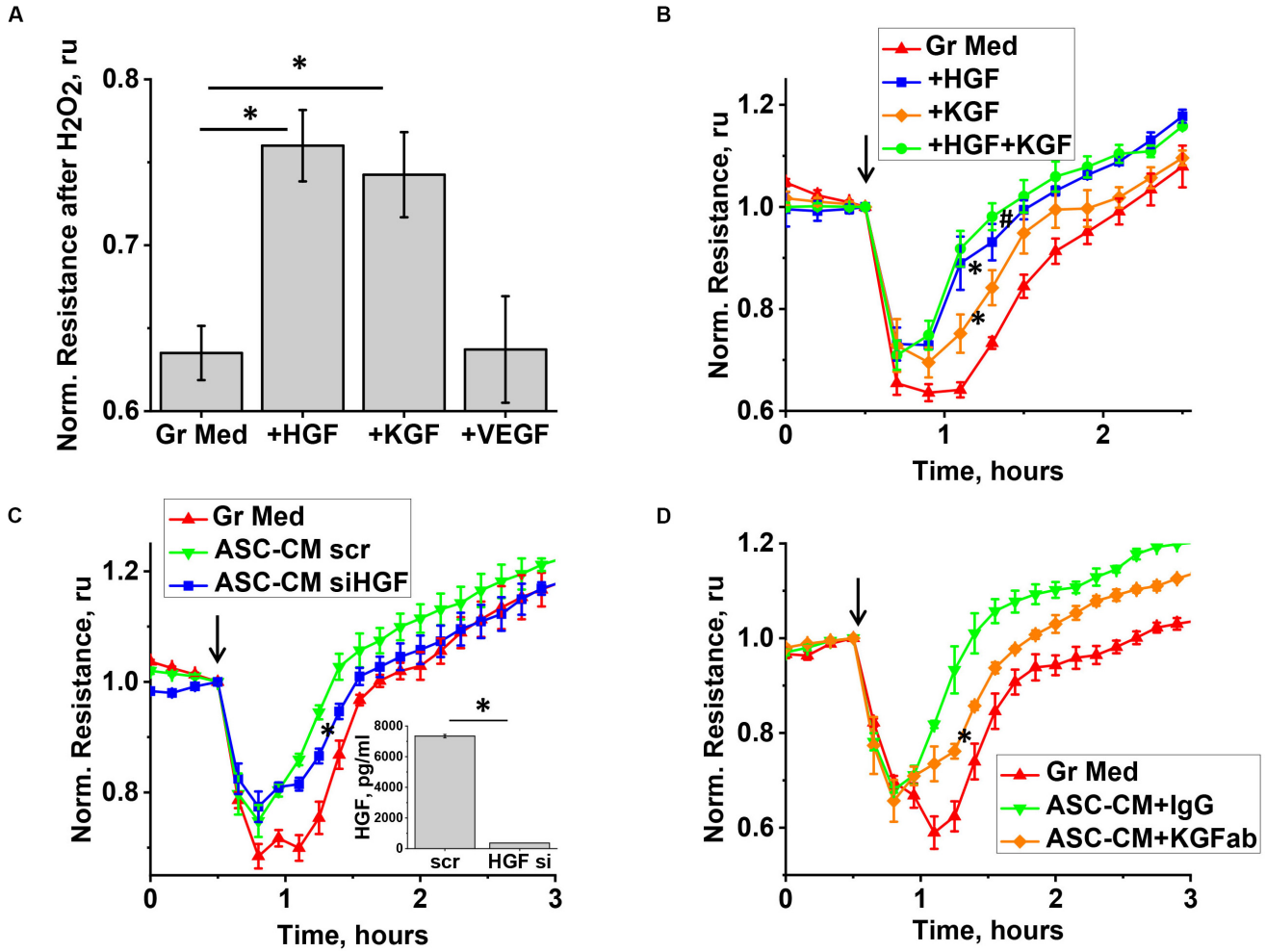

FIGURE 7 | KGF and HGF protect the endothelium from H_2O_2-induced barrier dysfunction. **(A,B)** HPAEC grown on gold electrodes of ECIS arrays were exposed to the original growth media (B-red), or growth media supplemented with KGF (B-orange), HGF (B-blue), KGF + HGF (B-green), or VEGF concentrations detected in ASC-CM conditioned for 48 h (see **Figure 6**). Media supplemented with factors were removed after 48 h of pretreatment, and HPAEC were challenged with 250 μM H_2O_2. For **(A)**, TER values were pooled from three independent experiments, with at least two parallel recordings each, and calculated as fold decrease in TER 30 min after H_2O_2 addition. **(C)** HPAEC grown on gold electrodes of ECIS arrays were exposed to the original growth media, or ASC-CM generated by ASC treated with scrambled siRNA, or HGF siRNA. Media were removed after 48 h of pretreatment, and HPAEC were challenged with 250 μM H_2O_2. Inset shows the concentration of HGF in ASC-CM collected from scrambled RNA-treated and HGF siRNA-treated ASC. **(D)** HPAEC grown on gold electrodes of ECIS arrays were exposed to the original growth media, or ASC-CM pre-incubated with control IgG or KGF neutralizing antibody. Media were removed after 48 h of pretreatment, and HPAEC were challenged with 250 μM H_2O_2. **(A)** *Differences detected with one-way ANOVA with Tukey *post hoc* ($p < 0.05$). **(B)** Repeated measurements ANOVA detected differences between responses of HPAEC exposed to growth media supplemented with either KGF or HGF (*), and the original growth media. Differences were also detected between responses of HPAEC exposed to media supplemented with KGF and KGF + HGF (#). **(C,D)** * Repeated measurements ANOVA detected differences between responses of HPAEC exposed to unmanipulated ASC-CM or ablated ASC-CM. (**C**, inset) * Differences were detected by *t*-test ($p < 0.05$).

these data suggested that carbon monoxide represents one of the factors conferring ASC-CM-mediated endothelial protection from apoptosis.

Protection of Endothelium From Apoptosis Is Dependent on c-Jun N-Terminal Kinase Inactivation

To learn about which pro-apoptotic pathways are targeted by apoptosis-protective factors in ASC-CM, we first analyzed endothelial MAP kinases activated in response to TNFα. We observed that TNFα stimulation leads to robust phosphorylation of p38 and JNK (**Figure 10A**) and very limited phosphorylation of ERK. However, when the effect of ASC-CM exposure was analyzed, only JNK phosphorylation was dramatically suppressed. Activation of JNK preceded activation of caspase 3 cleavage (**Figure 10B**), suggesting that JNK activation lays upstream of the activation of pro-apoptotic caspase cascades. To check if inhibition of JNK would protect from TNFα-induced apoptosis, we pretreated HPAEC with JNK inhibitor

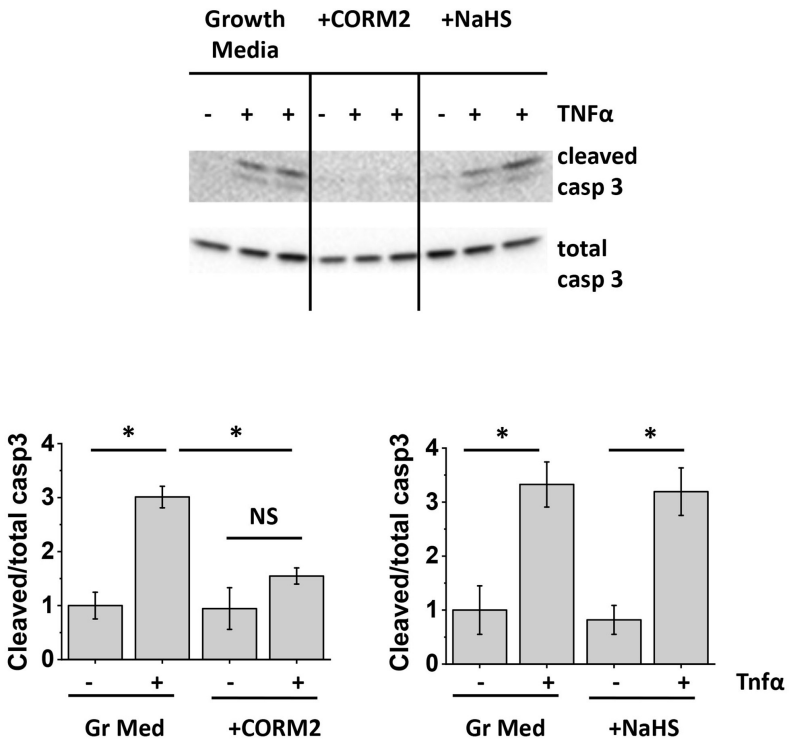

FIGURE 8 | Carbon monoxide protects the endothelium from apoptosis. HPAEC were exposed to the original growth media or growth media supplemented with 50 μM CORM2 (CO donor) or 50 μM NaHS (H_2S donor). After 48 h, media was removed; cells were stimulated with 2 ng/ml TNFα. Cell lysates were analyzed with antibodies to cleaved and total caspase 3. Data from three independent experiments were pooled and presented as cleaved/total caspase 3 ratio mean ± SEM. One-way ANOVA with Tukey *post hoc* was used to detect whether differences between indicated columns are significant *($p < 0.05$) or not significant (NS).

II. Indeed, we observed that in the presence of JNK inhibitor, cleavage of caspase 3 in response to TNF was significantly attenuated (**Figure 10C**).

To ascertain that the ASC-CM factors suppressing JNK activation are of the same nature as the factors suppressing caspase 3 activation, we first preconditioned HPAEC with a 3-kDa flow-through of ASC-CM. **Figure 11A** shows that 3 kDa flow-through effectively suppressed JNK phosphorylation, similar to suppression of caspase 3 cleavage shown previously (**Figure 2B**). We next verified whether xenogeneic ASC-CM would have an effect similar to allogeneic ASC-CM. Preconditioning of HPAEC with rat ASC-CM resulted in effective suppression of JNK phosphorylation (**Figure 11B**), suggesting that inhibition of the JNK pathway and inhibition of the pro-apoptotic pathway are rendered by a similar class of factors in ASC-CM. Finally, we preconditioned HPAEC with the CM from HO-1-depleted ASC and assessed JNK phosphorylation in response to TNFα. As was earlier shown with caspase 3 activation, CM from HO-1-depleted ASC displayed attenuated ability to suppress JNK activation (**Figure 11C**).

DISCUSSION

The progress of stromal cells to clinical studies testing application in multiple diseases highlighted several issues that may limit the widespread adoption for clinical use. These include issues with consistency of cell preparations for autologous treatments and logistical issues associated with cell storage, distribution, and bioactivity of cryopreserved cell preparations, whether autologous or allogeneic. Although closed system isolation devices, allowing isolation and readministration of autologous ASC-containing products at the point of care, are available for clinical use (Nordberg and Loboa, 2015), issues of patient-to-patient variability and safety concerns pertinent to the risk of thromboembolism remain (Toyserkani et al., 2017). The search for cell-free alternatives with potential advantages in safety, material handling, and bioactivity control was undertaken based on the discovery that stromal cell secretome manifested a significant portion of the effects attributed to stromal cells. The therapeutic potential of factors secreted by stromal cells was noted in multiple preclinical models of diseases, including acute conditions (Parekkadan et al., 2007; Cho et al., 2012; Ionescu et al., 2012; Lu et al., 2015; Pouya et al., 2018) for which the application of the off-the-shelf biologic can be of direct benefit as the product could be administered without delay and would exert immediate bioactivity, which may be critical in acute care settings such as stroke or myocardial infarction. To develop CM formulation translatable to clinical use, understanding of the effects of the manufacturing process on the product cost, logistics of storage and handling, and bioactivity is critically important. That is why, even though preclinical (Bi et al., 2007;

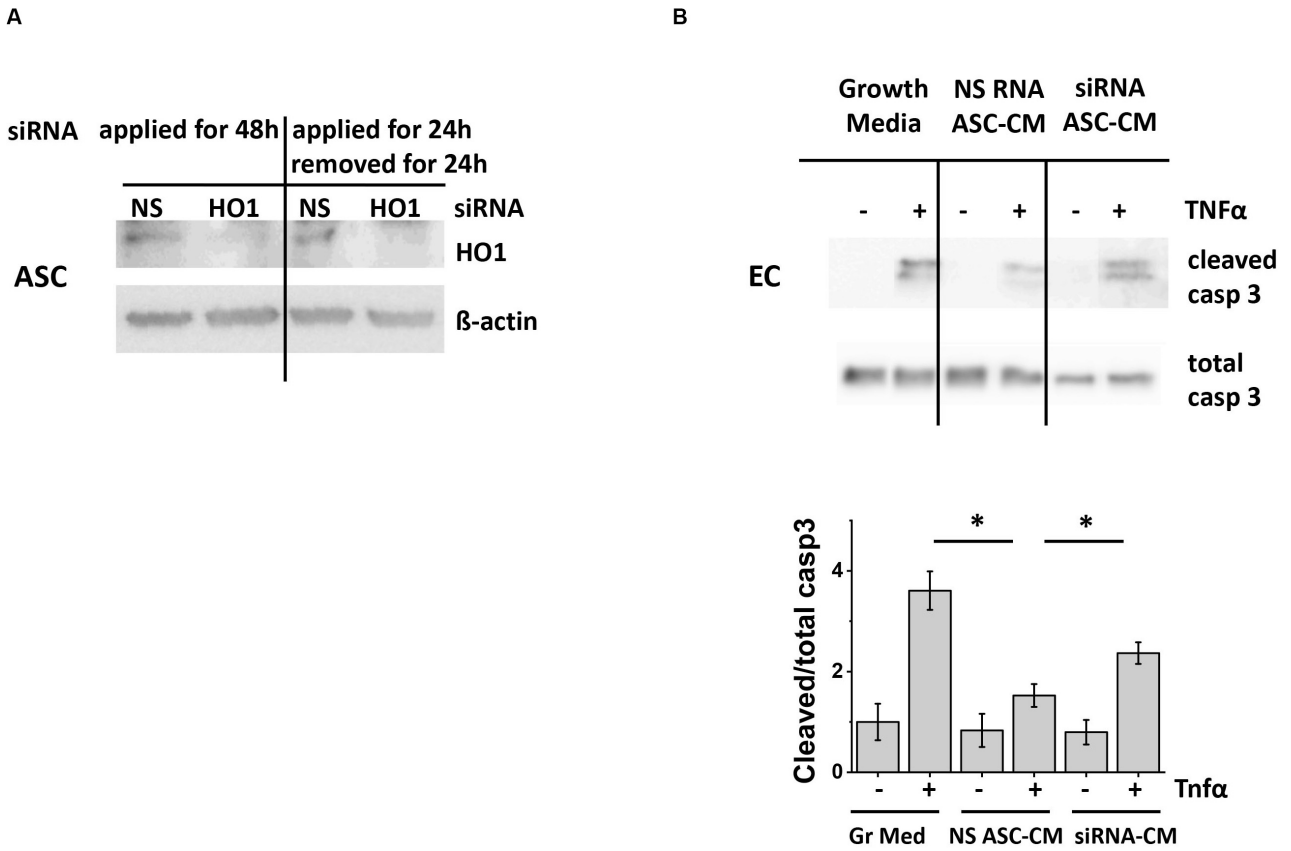

FIGURE 9 | ASC with suppressed heme oxygenase 1 expression generate ASC-CM with reduced apoptosis-protective potency. **(A)** Left: ASC were treated continuously with 50 nM of non-specific (NS) or heme oxygenase 1 (HO-1)-specific siRNA for 48 h. Right: Treatment was applied for 24 h, followed by a 24 h incubation in siRNA-free media. Cell lysates were analyzed with antibodies to heme oxygenase 1 and β-actin (loading control). **(B)** ASC-CM was generated from ASC treated for 24 h with 50 nM non-specific RNA (NS RNA) or heme oxygenase 1-specific siRNA (siRNA), followed by siRNA removal and 24 h conditioning with media. HPAEC pre-incubated for 48 h with growth media or ASC-CM were stimulated with 2 ng/ml TNF. Cell lysates were analyzed with antibodies to cleaved and total caspase 3. Data from three independent experiments were pooled and presented as cleaved/total caspase 3 ratio mean ± SEM. *Significance between TNF-stimulated groups assessed by *t*-test with Welch's correction as indicated ($p < 0.05$).

Yamagata et al., 2013; Suto et al., 2016) and some clinical (Zhou et al., 2013; Dahbour et al., 2017) studies show the ability of unconcentrated CM to exert beneficial effects and limit pathological conditions, significant effort is devoted to analyze the effects of CM lyophilizates (Fukuoka et al., 2017; Katagiri et al., 2017) and EV concentrates (Kordelas et al., 2014; Nassar et al., 2016). Formulation of the particular therapeutic is likely to be optimized for specific clinical use; therefore, elucidation of the nature of bioactive factors is of utmost importance for the preservation of therapeutic potential during the manufacturing process and will be of utmost significance to gaining regulatory approval. Here, we used an unbiased approach to analyze biological activities in different fractions of the original CM, to gain information about the nature of bioactive factors and, most importantly, aid in understanding how routine procedures used in CM manufacturing, such as concentration utilizing different MW filters, can affect the biological activity of the CM product.

This study is not the first attempt to identify the fraction of stromal cell CM with specific biologic activity. As one can expect, the MW of active fraction depends on the nature of activity for which it is tested for. For example, the ability to preserve pancreatic islets was assigned to 10–30 and > 50 kDa fractions of ASC-CM (Kasahara et al., 2013). The ability to promote macrophage shift to anti-inflammatory phenotype was assigned to < 3 and 50–100 kDa fractions of CM (Ylostalo et al., 2012). On the contrary, the ability to reduce myocardial infraction was seen only in the CM fraction of > 1,000 kDa (Timmers et al., 2007). To aid in understanding endothelial-specific effects with the potential to apply this knowledge for treatment of vascular leak and endothelial damage associated with acute lung injury, we used endothelial barrier dysfunction and apoptosis as two easily quantifiable readouts. Employing human pulmonary endothelial cells as a reporting system, we assessed (1) barrier dysfunction in response to H_2O_2, mimicking endothelial response to oxidative burst, and (2) activation of pro-apoptotic pathways in response to TNFα, mimicking endothelial apoptotic response to cytokine storm. Although an early work (Petrache et al., 2003) failed to detect significant HPAEC apoptosis in response to TNFα, later studies showed consistent activation of pro-apoptotic caspase 3 (Lu et al., 2015) and dramatic increase in TUNEL-positive

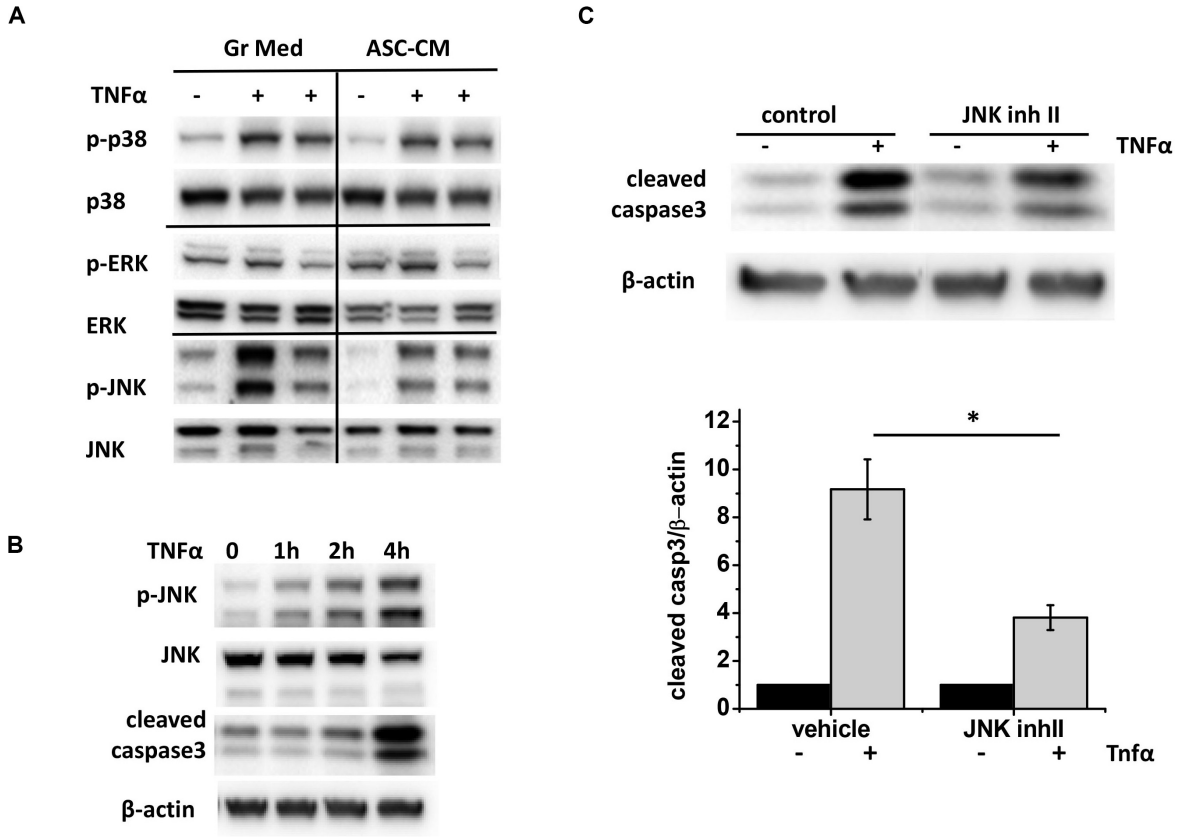

FIGURE 10 | ASC-CM exposure suppresses JNK activation, which precedes and contributes to caspase activation in response to TNFα in the endothelium. **(A)** HPAEC exposed to growth media or ASC-CM were challenged with 2 ng/ml TNFα for 4 h. Cell lysates were analyzed with antibodies to phospho- and pan-p38, phospho- and pan-ERK, and phospho- and pan-JNK. **(B)** HPAEC were stimulated with 2 ng/ml TNFα for the times indicated. Cell lysates were analyzed with antibodies to p-JNK, JNK, cleaved caspase 3, and β-actin (loading control). **(C)** HPAEC pretreated with vehicle control or 5 μM JNK inhibitor II for 30 min were treated with 2 ng/ml TNFα for 4 h. Data from three independent experiments were pooled and presented as cleaved caspase 3/β-actin ratio, normalized to no TNF control. *t-test with Welch's correction was used to detect significant differences ($p < 0.05$).

HPAEC in response to TNFα (Koh et al., 2007; Bae and Rezaie, 2008). Using readouts of H_2O_2-induced barrier dysfunction and TNFα-induced caspase 3 cleavage in HPAEC treated with different fractions of ASC-CM, we showed that barrier-protective and apoptosis-protective activities of ASC-CM are carried by its two distinct fractions, > 50 and < 3 kDa, respectively.

Analysis of the potential involvement of HGF and KGF, earlier shown to be secreted by stromal cells (Cai et al., 2007; Lafosse et al., 2016) and known to affect barrier function (Gillis et al., 1999; Liu F. et al., 2002), revealed that (1) these factors fractionate with MW exceeding 100 kDa and (2) levels of factors present in ASC-CM are sufficient to confer barrier resistance to H_2O_2 upon preconditioning. Ablation of either factor with neutralizing antibody or siRNA knockdown rendered CM with attenuated ability to protect the endothelium. Although the levels of HGF and KGF determined in CM by ELISA were not affected by CM centrifugation and therefore represented the levels of soluble factors, we cannot exclude the existence of exosome-associated factors with similar biologic activity. Consistently, the barrier-protective activity of CM can be partially attributed to extracellular vesicle/exosome fraction of CM. This fraction is also known to contain vast cargo of bioactive proteins and microRNA, whose particular analysis lies beyond the scope of this study.

Examination of the apoptosis-protecting activity of CM revealed heat-insensitive, non-species-specific, and small MW nature of these factors. Our attention was drawn to the possible role for the secreted gases; two of them, hydrogen sulfide and carbon monoxide, are known to be stable and anti-apoptotic (Almeida et al., 2012; Wu et al., 2015). Preconditioning of the endothelium with donors of H_2S and CO revealed that only CORM2, the CO donor, is able to protect the endothelium from TNFα-induced apoptosis. In the absence of direct methods to measure CO production in CM, our next step was to ablate the CO-generating enzyme, heme oxygenase 1, in ASC. Our data showed that knockdown of heme oxygenase 1 decreased the apoptosis-protective potency of ASC-CM, suggesting that carbon monoxide generation by ASC is important for the anti-apoptotic activity exhibited by ASC or ASC unmanipulated secretome.

Further examination into the apoptosis-mediating mechanisms affected by CM preconditioning revealed the causative nature of JNK phosphorylation in the endothelium. The inhibitor used in the study is known to suppress both

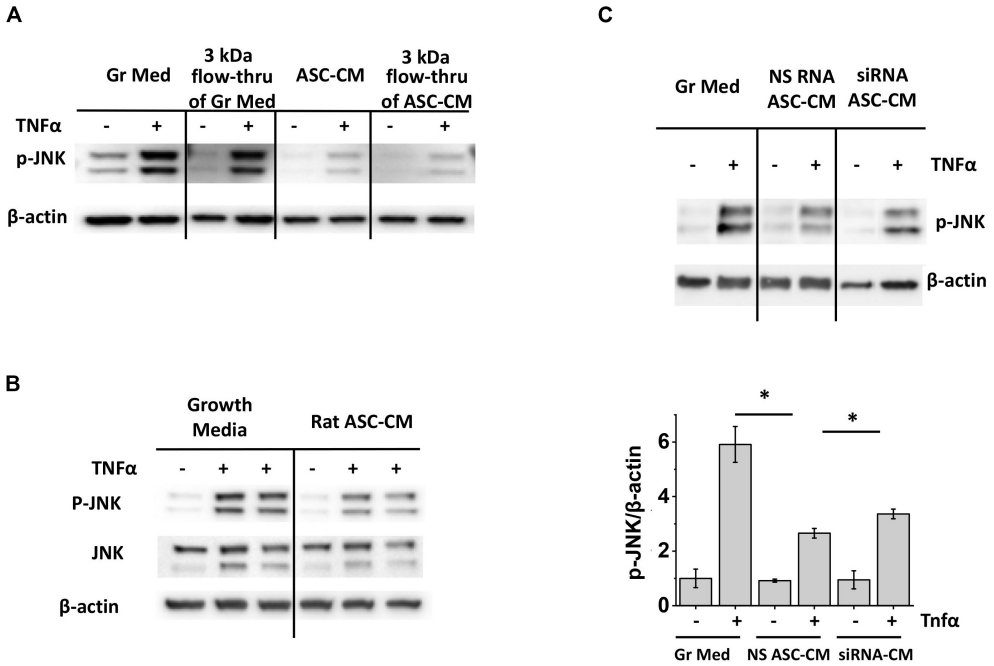

FIGURE 11 | Inhibition of JNK activation is mediated by small MW factors in ASC-CM which are not species-specific. Depletion of heme oxygenase 1 in ASC attenuates ASC-CM ability to inhibit JNK activation. **(A)** HPAEC pretreated with the original growth media and ASC-CM or the 3 kDa flow-through fractions of growth media and ASC-CM were challenged with 2 ng/ml TNFα for 4 h. **(B)** HPAEC pretreated with growth media or rat ASC-CM were challenged with 2 ng/ml TNFα for 4 h. **(C)** HPAEC pretreated with growth media or ASC-CM generated by non-specific RNA-treated ASC (NS RNA ASC-CM) or heme oxygenase 1 siRNA-treated ASC (siRNA ASC-CM) were challenged with 2 ng/ml TNFα for 4 h. Cell lysates were analyzed with antibodies to p-JNK and β-actin (loading control). Data from three independent experiments were pooled and presented as cleaved caspase 3/β-actin ratio mean ± SEM. *Significance between TNF-stimulated groups assessed by *t*-test with Welch's correction as indicated ($p < 0.05$).

JNK1 and JNK2 activity. A recent study showed that only JNK1 possesses pro-apoptotic activity in the lung (Tan et al., 2020). Importantly, TNFα-induced JNK phosphorylation was suppressed by ASC-CM in the same heat-insensitive, non-species-specific, and low MW factor-dependent manner as TNFα-induced caspase 3 activation, prompting the examination of the role of carbon monoxide. Finally, we had shown that knockdown of heme oxygenase 1 in ASC reduced ASC-CM's ability to inhibit JNK phosphorylation. Altogether, these data helped pinpoint carbon monoxide as one of the factors contributing to ASC-CM's anti-apoptotic activity and delineate the pro-apoptotic pathways attenuated by ASC-CM in the endothelium. One of the limitations of the study is that the nuclear factor kappa B (NF-κB) pathway, contributing to TNFα response (Kempe et al., 2005), was not studied, whereas this pathway is known to protect cells from apoptotic death, in particular, by downregulating JNK signaling (De Smaele et al., 2001). Previously, proteomic analysis of exosomes released from serum-starved and oxygen-deprived MSC showed enrichment in NF-κB-regulating nodes; in the same study, MSC's exosome ability to induce endothelial angiogenesis was shown to be NF-κB-dependent (Anderson et al., 2016). As our study shows that protection from apoptosis is mediated by the exosome-free fraction of ASC-CM, it would be interesting to see how TNFα-induced endothelial NF-κB signaling is affected by ASC-CM manipulation.

Summarizing the impact of our data for the development of application-specific CM preparations, we have to emphasize that although the barrier-protective activity of CM will be preserved in preparations generated by the concentration with low MW cutoff filters or ultracentrifugation, a significant proportion of apoptosis-protective activity will be lost. Nonetheless, the concentration of CM is the most likely scenario to be followed by large-scale manufacturers for clinical purposes; therefore, emphasis of future studies should be on the barrier-protective and immunomodulatory activities of fractions preserved after the concentration of CM with low MW cutoff filters. In the context of our data, standardization criteria for clinical applications aiding to preserve endothelial barrier should include assessment of levels of HGF and KGF in CM preparations. Our data regarding low MW components of secretome with anti-apoptotic activity, although of limited relevance to the development of CM concentrates, can be used to develop tests assessing the therapeutic potential of stromal cells intended for cellular therapy.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/supplementary material.

AUTHOR CONTRIBUTIONS

KM, MC, and NB contributed to the conception and design of the study. HL and NB carried out the majority of the experiments and statistical analyses. SM-C and YJ contributed to the experimental work. NB wrote the first draft of the manuscript. All authors contributed to manuscript revision and read and approved the submitted version.

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Conflict of Interest: The authors declare that there is intellectual property granted to KM and NB for the treatment of ARDS with ASC-CM and that Theratome Bio, Inc. has a business interest in this intellectual property.

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REVIEW
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Extracellular Vesicle-Dependent Communication Between Mesenchymal Stromal Cells and Immune Effector Cells

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Mesenchymal stem/stromal cells (MSCs) are multipotent cells residing in the stromal tissues of the body and capable of promoting tissue repair and attenuating inflammatory processes through their immunomodulatory properties. Preclinical and clinical observations revealed that not only direct intercellular communication mediates MSC properties; in fact, a pivotal role is also played by the release of soluble and bioactive factors, such as cytokines, growth factor and extracellular vesicles (EVs). EVs are membrane-coated vesicles containing a large variety of bioactive molecules, including lipids, proteins, and nucleic acids, such as RNA. EVs release their contents into target cells, thus influencing cell fate through the control of intracellular processes. In addition, MSC-derived EVs can mediate modulatory effects toward different effector cells belonging to both innate and adaptive immunity. In this review, we will discuss the literature data concerning MSC-derived EVs, including the current standardized methods for their isolation and characterization, the mechanisms supporting their immunoregulatory properties, and their potential clinical application as alternative to MSC-based therapy for inflammatory reactions, such as graft-versus-host disease (GvHD).

Keywords: extracellular vesicles, exosomes, microvesicles, mesenchymal stromal cells, immune effector cells, immunomodulation

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INTRODUCTION

Mesenchymal stromal cells (MSCs) are multipotent stem cells of mesodermal origin described in bone marrow (BM) for the first time by Alexander Friedenstein in 1966 (Friedenstein et al., 1966). Over the last decades, MSCs were also identified in a large number of tissues, including fat, umbilical cord, amniotic fluid, placenta, skin, dental pulp, and many others (Riekstina et al., 2008; Marquez-Curtis et al., 2015; Camilleri et al., 2016; Ventura Ferreira et al., 2018; Caseiro et al., 2019; Fukutake et al., 2019). In 2006, the International Society for Cellular Therapy (ISCT) established the minimal criteria to define human MSCs, i.e., (i) plastic-adherence when maintained in standard culture conditions; (ii) surface expression of CD105, CD73 and CD90 antigens while lacking CD45, CD34, CD14 or CD11b, CD79α or CD19, and HLA-DR molecules; (iii) *in vitro* differentiation into three mesodermal lineages (osteoblasts, adipocytes, and chondrocytes) (Dominici et al., 2006).

MSCs boosted a great interest in the field of regenerative medicine and tissue engineering thanks to their ability to promote tissue regeneration and to modulate immune response (de Mayo et al., 2017; Petri et al., 2017; Pokrywczynska et al., 2019). Indeed, MSCs possess broad immunomodulatory properties affecting immune effector cells of both innate and adaptive responses (Krampera, 2011). For example, MSCs are capable of stimulating cytokine release and proliferation of innate lymphoid cells (van Hoeven et al., 2018), affecting dendritic cell (DC) maturation and activation (Zhang et al., 2004), suppressing natural killer (NK) cell activity and proliferation (Spaggiari et al., 2008), supporting the expansion of myeloid-derived suppressor cells (MDSCs) (Yen et al., 2013), and regulating B cell proliferation and activation (Fan et al., 2016) as well as T cell activity, balance between T helper (Th)1 and Th2 lymphocytes and expansion of T regulatory (Treg) cells (Haddad and Saldanha-Araujo, 2014; Gao et al., 2016). The ability of MSCs to modulate the immune response is well documented by several preclinical and clinical studies in a wide range of inflammatory and autoimmune diseases, such as Crohn's disease (Forbes, 2017), rheumatoid arthritis (Ansboro et al., 2017), diabetes (Cho et al., 2018), graft-versus-host disease (GvHD) (Le Blanc et al., 2008), sepsis (Hall et al., 2013), cardiovascular diseases (Bagno et al., 2018), allergic airway inflammation (Takeda et al., 2018), and many others. Initially, the biological activity of MSC was ascribed to their ability to home within the injury site; however, only a small fraction of MSCs is capable of reaching the damaged tissues after systemic administration (Kraitchman et al., 2005; Yukawa et al., 2012; Scarfe et al., 2018), while the majority of them are rapidly cleared through phenomena of efferocytosis, thus polarizing macrophages toward an inhibitory phenotype (Galleu et al., 2017). In addition, MSCs may act at paracrine level through the release of bioactive factors, including transforming growth factor β (TGF-β), hepatocyte growth factor, prostaglandin E2 (PGE2), interleukin (IL)-10 and IL-6, human leukocyte antigen G (HLA-G), indoleamine-2,3-dioxygenase (IDO), nitric oxide (NO), and other mediators (Sato et al., 2006; Ryan et al., 2007; Németh et al., 2009; Bouffi et al., 2010; Du et al., 2016; Wang et al., 2018; Liu et al., 2019; Lu et al., 2019; Pittenger et al., 2019). In the last years, membrane-bound particles, known as extracellular vesicles (EVs), have been recognized as an important MSC paracrine factor in addition to soluble factors (Chen et al., 2016; Bier et al., 2018). EVs represent a very effective, physiological intercellular communication, even at low molecule concentrations at which soluble factors could be rapidly inactivated. Strong experimental evidence shows that MSC-EVs are capable of recapitulating the immunomodulation of their parental cells (Rani et al., 2015; Seo et al., 2019). Therefore, in this review we will provide an overview of the literature data supporting the MSC-EV-dependent communication between MSCs and immune effector cells (IECs).

CHARACTERIZATION OF EVs

EVs consist of a phospholipid bilayer envelope acting as molecular shuttle for various molecules, such as proteins, different types of nucleic acids, lipids and active metabolites (Lai et al., 2016; Yuan et al., 2017; Yang et al., 2018; Shojaati et al., 2019). Historically, EVs are classified into three main groups according to their biogenesis and size: (i) exosomes, (ii) microvesicles and (iii) apoptotic bodies. Exosomes (diameter range 50–100 nm) represent the smallest EV fraction deriving from the fusion of intracellular endosomes with plasma membrane, followed by their release into the extracellular space (Stephen et al., 2016). The production of exosomes is generally constitutive, although it can increase upon cell stimulation (Fierabracci et al., 2015). Microvesicles (MVs; diameter range 100–1,000 nm) are generated by cytoplasmic membrane budding in response to several stimuli resulting in cytosolic Ca^{2+} increment and disassembly of the cytoskeleton (Ratajczak et al., 2006). Apoptotic bodies (diameter range 1–5 μm) are characterized by irregular shapes and heterogenous sizes (Caruso and Poon, 2018). Apoptotic bodies are functionally different, as they are released during apoptosis and contain mainly cellular debris, such as micronuclei, chromatin remnants and cytosol portions (Battistelli and Falcieri, 2020). As several studies were performed with different separation approaches and cellular sources of EVs, it is still not possible to propose a specific classification of different EV subtypes as well as specific markers and biogenesis processes (Gould and Raposo, 2013; Cocucci and Meldolesi, 2015). Consequently, the Minimal Information of Studies of Extracellular Vesicles 2018 (MISEV2018) suggests to use the generic terms "small/medium/large EVs," according to their size or density, instead of the classical "exosomes," "microvesicles," and "apoptotic bodies" terms (Théry et al., 2018). According to MISEV2018, to confirm the nature of EVs and the degree of purity of EV preparation, the scientific community has encouraged to evaluate the presence of at least one of transmembrane or GPI-anchored proteins associated to plasma membrane and/or endosomes (for example tetraspanins, integrins, and MHC class I) and cytosolic proteins recovered in EVs (for example lipid or membrane protein-binding ability like ESCRT-I/II/III and ALIX or promiscuous proteins like HSP70 or cytoskeleton proteins like actin and tubulin) and major components of non-EV co-isolated structures (for example lipoproteins, protein/nucleic acid aggregates, and ribosomal proteins) (Théry et al., 2018). Additionally, for studies focused on one or more EV subtypes is recommended to assess the presence of transmembrane, lipid-bound and soluble proteins associated to other intracellular compartments than plasma membrane/endosomes, including lamin A/C, cytochrome C, calnexin, and ATG9A, whereas for the evaluation of EV functional activities, the identification of functional soluble factor in EVs like cytokines, growth factors, adhesion and extracellular matrix proteins is required (Théry et al., 2018).

The communication system based on EVs is highly conserved among the three different animal reigns, thus suggesting how EVs are crucial for intercellular communication (Deatherage and Cookson, 2012; Gill et al., 2019). EVs contribute to cell-to-cell communication via direct contact with target cells through a ligand–receptor interaction. In particular, EVs can transfer information to target cells either without delivering their content or acting like biological shuttles that release their

cargo into acceptor cells. A classic example of EV contribution to intercellular communication without deliver their content resides in those vesicles that harbor MHC molecules on their surface, thus activating T cell receptors on T cells (Raposo et al., 1996; Martin et al., 2014). Concerning the delivery of EV content, EVs can be taken up by target cells through several mechanisms, including clathrin-mediated endocytosis, caveolin-dependent endocytosis, macropinocytosis, phagocytosis, lipid rafts, and cell surface membrane fusion (Feng et al., 2010; Montecalvo et al., 2012; Svensson et al., 2013; Tian et al., 2014; Costa Verdera et al., 2017; Rai and Johnson, 2019). Although numerous receptors/ligands are implicated into EV uptake including tetraspanins, integrins, immunoglobulins, lectins, and proteoglycans (Morelli et al., 2004; Hao et al., 2007; Barrès et al., 2010; Christianson et al., 2013), to date it is still debated whether EV uptake is a cell-type specific process or not. Indeed, some studies suggest that EVs from different sources can be taken up by every cell type (Costa Verdera et al., 2017; Horibe et al., 2018), whereas others report that only a particular combination of EV and target cells (and thus the right association between receptors and ligands) allow the EV uptake by acceptors cells (Fitzner et al., 2011; Zech et al., 2012; Chivet et al., 2014; Di Trapani et al., 2016). Finally, recent evidence suggested that nanotubes could synergistically act with EVs in intercellular communication, as microsized particles could be transferred into target cells via nanotubes (Ware et al., 2015; Nawaz and Fatima, 2017).

As EVs reflect the characteristics of their cells of origin both at molecular and functional level, EVs have emerged as a novel potential therapeutic approach due to their ability to influence various biological processes, including immune response, cell proliferation, tissue regeneration, cell invasiveness, tubule formation, angiogenesis, synapsis plasticity, and many others (Zaborowski et al., 2015; Silva et al., 2017; Prada et al., 2018; Lee et al., 2019; Mou et al., 2019).

MSC-EVs AND IMMUNOMODULATION

MSC-EVs play a pivotal role in mediating the paracrine effects of MSCs on immune system. Generally, MSC-EVs may promote an immunosuppressive response through the induction of immature DCs, the polarization of macrophages toward M2-like phenotype, the inhibition of immunoglobulin (Ig) release, the expansion of Tregs and the secretion of anti-inflammatory cytokines (Budoni et al., 2013; Burrello et al., 2016; Favaro et al., 2016; Balbi et al., 2017; Du et al., 2018). However, MSC-EVs should be considered in the whole contest of MSC secretome, because in some experimental settings the immunomodulation mediated by MSC-EVs can only poorly recapitulate the immune properties of their parental cells (Conforti et al., 2014; Gouveia de Andrade et al., 2015; Ma et al., 2019). In the next sections we will try to give a comprehensive overview of the effects of MSC-EVs on the innate (macrophages, DCs and NK cells) and adaptive (B and T cells) immune system. As the studies here reported employed different EV subtypes obtained from several MSC sources (BM, umbilical cord, adipose tissue, fetal liver) of different animal species (human, mouse and rat) with several isolation methods, we will refer to them with the generic term "MSC-EVs." The immunomodulatory effects of MSC-EVs on innate and adaptive immune system are summarized in **Figure 1**.

MSC-EVs and Innate Immune System

Macrophages

Macrophages are mononuclear phagocytes with important roles in physiological conditions and in first-line immune response (Zhang and Wang, 2014). Macrophages are extremely plastic cells, with the capability of differentiating into two activated subtypes, i.e., M1 and M2. M1 macrophages are classical activated cells secreting large amount of pro-inflammatory factors, such as TNF-α, IL-1β and reactive oxygen species. On the other hand, M2 macrophages are alternatively activated and anti-inflammatory cells producing IL-10 and trophic factors (Shapouri-Moghaddam et al., 2018). Recent data support a contribution of MSC-EVs in modulating the M1/M2 balance, although the precise mechanism remains unclear. For instance, MSC-EVs may hamper the activation of pro-inflammatory M1 macrophages in favor of pro-resolving M2 macrophages that parallel with VEGF-A, IFN-γ, IL-12, and TNF-α reduction as well as IL-10 upregulation (Balbi et al., 2017; Cosenza et al., 2017; Cao et al., 2019). The modulation of several signaling pathways mediated by MSC-EVs may be responsible for this effect. For instance, the inhibition of JAK/STAT signaling was confirmed by many studies, resulting in Arg1 increment and inflammation reduction (Zhao et al., 2018; Cao et al., 2019). The activation of S1P/SK1/S1PR1 signaling by MSC-EVs promotes M2 differentiation through the downregulation of NF-κB-p65 and TGF-β1 expression in macrophages, thus restoring cardiac activity after myocardial infarction (Deng et al., 2019). Furthermore, lipopolysaccharide (LPS)-primed MSC-EVs support M2 macrophage polarization, by interfering with LPS−dependent NF−κB signaling, and partly activate the AKT1/AKT2 signaling pathway, by attenuating the post-infarction inflammation and cardiomyocyte apoptosis (Xu et al., 2019). An interesting mechanism by which MSC-EVs exert their anti-inflammatory function is the M2 polarization through MSC-EV-mediated mitochondrial transfer that is dependent on macrophage oxidative phosphorylation (Morrison et al., 2017). In parallel, in response to oxidative stress, MSCs outsource mitochondria depolarized by MVs, thus enhancing macrophage bioenergetics (Phinney et al., 2015) and therefore their pro-inflammatory features (Tavakoli et al., 2013). Moreover, MSCs-EVs may trigger the anti-inflammatory phenotype and pro-resolving properties of mature, human regulatory macrophages, a subclass of M2 macrophages characterized by modest IL-22 and IL-23 production and PGE2 hyper-expression, thus leading to reduction of Th17 response (Hyvärinen et al., 2018). MSC-EVs can also reduce chemokine expression (CXCL1 and CCL5) that are necessary for inflammatory response by macrophages (Zou et al., 2014; Willis et al., 2017). Interestingly, MSC-EVs express CCR2 chemokine, bind and reduce the concentration of the free pro-inflammatory CCL2 ligand, and therefore prevent the activation and recruitment of M1 macrophages (Shen et al., 2016). MSC-EVs may also trigger the

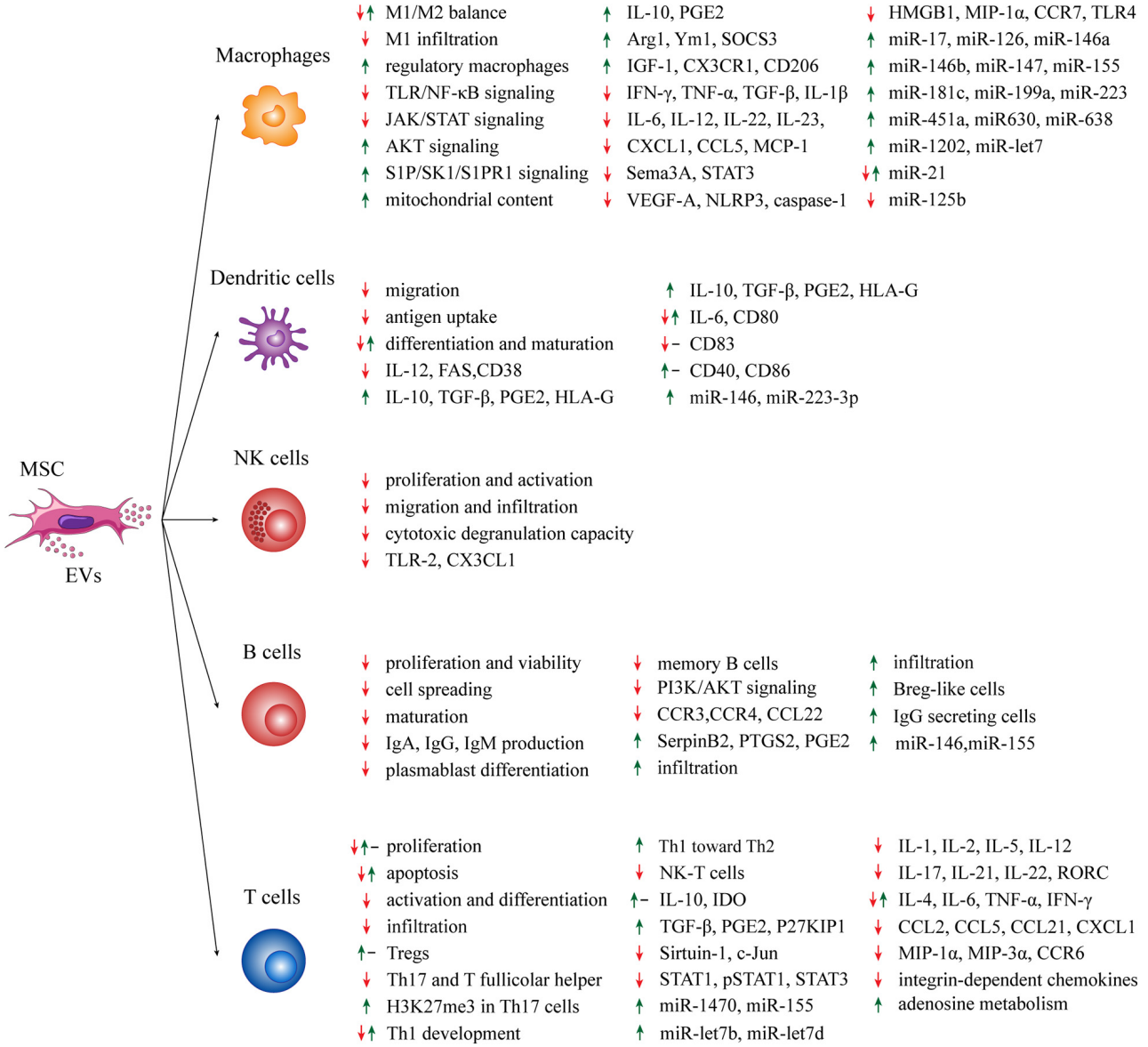

FIGURE 1 | Summary of the effects of MSC-EVs on immune effector cells. Green arrows: positive effect; red arrows: negative effect; black dashes: no effect.

anti-inflammatory phenotype in hepatic macrophages through IGF-1 (Fiore et al., 2020).

Several miRNAs are involved into MSC-EV-mediated anti-inflammatory effects on M1/M2 balance. For instance, miR-223 overexpression in MSC-EVs may reprogram macrophages from M1 to M2 phenotype by targeting Sema3A and STAT3 (Wang et al., 2015; He et al., 2019). Under hypoxic condition, the enrichment of miR-223 determines the overexpression of miR-146b, miR-126 and miR-199a, which in turn upregulate the expression of Arg1 and Ym1 and promote the anti-inflammatory M2 state (Lo Sicco et al., 2017). Other miRNAs involved in M2 polarization are miR-155 and miR-21, whose downregulation results in the increase of SOCS3 and M2 molecules (IL-10, CD206 and arginase) as well as M1 marker reduction (CCR7, IL-1β, IL-6, and NO) (Henao Agudelo et al., 2017). IL-1β-primed MSC-EVs express high levels of miR-146a promoting M2 macrophage polarization more effectively than IL-1β-primed MSC, thus increasing survival of septic mice (Song et al., 2017). The comparative miRNA analysis of EVs isolated from either IFN-γ-primed or resting MSCs revealed that miR-150-5p, whose target genes is involved in acute-phase response and signaling in macrophages, is downregulated in IFN-γ-primed EVs. Nevertheless, no difference between primed and resting EVs has been observed in promoting macrophage differentiation toward M2 phenotype (Marinaro et al., 2019). The enrichment of miR-let7 within MSC-EVs may favor M2 polarization and suppress macrophage infiltration through miR-let7/HMGA2/NF-κB pathway and miR-let7/IGF2BP1/PTEN

pathway, respectively (Li et al., 2019). Moreover, LPS-primed MSC-EVs regulate the M1/M2 macrophage balance more efficiently than resting EVs, thanks to the expression of miR-let-7b, which inhibits TLR4/NF-κB/STAT3/AKT signaling pathway, thus hampering inflammation and enhancing diabetic cutaneous wounds healing (Ti et al., 2015). TLR/NF-κB signaling can be targeted by other miRNAs too. In a model of burn-induced inflammation, the administration of MSC-EVs overexpressing miR-181c reduced the number of macrophages (and neutrophils) potentially inhibiting TLR4 expression and its downstream target proteins NF-κB/P65 and p-65, thus preventing inflammation (Li et al., 2016). MSC-EV fraction is characterized by the enrichment in miR-451a, miR-1202, miR-630, and miR-638 and the reduced expression of miR-125b and miR-21. This miRNA profile may be responsible for targeting MYD88-dependent inflammatory nodes to suppress TLR/NF-κB signaling pathway and macrophage activation (Phinney et al., 2015). Additionally, Zhang et al. found *in vitro* that MSC-EVs induce monocytes to switch toward an anti-inflammatory M2-phenotype via MYD88-dependent TLR signaling pathway, resulting in a reduction of IL-1β, IL-6, IL-12, and TNF-α levels and higher IL-10 concentration, thus polarizing activated $CD4^+$ T cells toward Treg subset (Zhang et al., 2013).

MSC-EVs also prevent M1-type macrophage infiltration in injury sites by lowering MCP-1, CCL5, HMGB1, and MIP-1α expression (Yu et al., 2016; Spinosa et al., 2018; Woo et al., 2020), probably through miR-147 expression (Spinosa et al., 2018). Interestingly, in a model of thioglycolate-induced peritonitis, treatment with MSC-EVs reduced macrophage infiltration in the peritoneal cavity by inducing a M2-like regulatory phenotype; this effect was partially associated to the upregulation of CX3CR1 in $F4/80^+/Ly6C^+/CCR2^+$ macrophage subset (Henao Agudelo et al., 2017). Concerning the homing ability of MSC-EVs, Lankford et al. demonstrated in a model of damaged spinal cord that MSC-EVs can migrate only in the injury site and M2-type macrophage are the primary target of EVs (Lankford et al., 2018).

Finally, MSC-EVs mediate miR-17 transfer from parental cells to macrophages, thus suppressing NLRP3 inflammasome activation, and consequently caspase-1, IL-1β, and IL-6, by targeting TXNIP (Liu et al., 2018). The suppression of NLRP3, caspase-1, IL-1β, and IL-6 was also reported by other authors (Jiang et al., 2019). On the other hand, EVs isolated from LPS-primed periodontal ligament stem cells (characterized by MSC-like markers) may induce strong M1-type polarization in association with pro-inflammatory molecules (TNF-α and IL-6); this effect seems related to double-strand DNA on EV surface (Kang et al., 2018).

Dendritic Cells

DCs are innate professional antigen-presenting cells (APCs) acting as central regulators of the adaptive immune response. DCs can be found in either resting or active state. Resting DCs are immature APCs expressing low levels of costimulatory molecules (CD38, CD40, CD80, CD83, and CD86) and immunostimulatory cytokines conferring high capacity to capture antigens. DC activation and maturation depend on different stimuli deriving from bacteria, viruses and damaged tissue. Activated DCs are potent T cell response inducers showing low antigen capture activity and high expression of histocompatibility complex II (MHC class II), costimulatory signals, C-C chemokine receptor type 7 (CCR7) as well as immunostimulatory cytokines (Collin et al., 2013; Patente et al., 2019). EVs secreted by different types of MSCs exert immunosuppressive effects on DCs primarily by inhibiting their activation, eventually leading to the lack of T cell response triggering. For example, DCs from type 1-diabetic (T1D) patients treated with heterologous MSC-EVs acquired an immature phenotype, characterized by low expression of activation markers and higher production of IL-6, IL-10, TGF-β, and PGE2 (Favaro et al., 2016). Therefore, MSC-EV-treated DCs inhibit the inflammatory T cell response by decreasing Th17 subset and inducing $Foxp3^+$ Tregs (Favaro et al., 2016). Similarly, MSC-EV treatment leads to anergic, IL-10-expressing, regulatory DCs that suppress Th1 and Th17 cell development, but without inducing Tregs (Shigemoto-Kuroda et al., 2017). Notably, MSC-EVs may enhance the release of TGF-β and IL-10 from $CD11c^+$ DCs, thus inhibiting lymphocyte proliferation, without affecting the expression of MHC class II, CD86, CD83, and CD40 (Shahir et al., 2020). Upregulation of miR-146 expression in DCs is a possible mechanism by which MSC-EVs promote DC immature phenotype, leading to the downregulation of FAS expression and IL-12 production (Wu et al., 2017). Alternatively, EVs derived from renal, mesenchymal-like cancer stem cells impair dendritic differentiation and T cell activation by upregulating the expression of the anti-inflammatory molecule HLA-G (Grange et al., 2015). MSC-EVs may also prevent immature DCs from antigen uptake by blocking their maturation (Reis et al., 2018). As a consequence, MSC-EVs lower CD38, CD80, CD83, IL-6, and IL-12 expression, increase the production of the anti-inflammatory cytokine TGF-β and reduce DC ability to migrate toward CCL21, the CCR7-ligand, although DCs can still trigger allogeneic T cell proliferation *in vitro* (Reis et al., 2018). These MSC-EV-treated DCs resulted enriched of four microRNAs (miR-21-5p, miR-142-3p, miR-223-3p, and miR-126-3p) mediating well-known effects on DC maturation and functions (Reis et al., 2018). On the other hand, higher expression of costimulatory factors (CD40, CD80, and CD86), but not MHC class II, can be observed on the surface of murine immature DCs following MSC-EV treatment, thus suggesting that these EVs can mediate the DC maturation required for the induction of effector T-cell (Cho et al., 2019).

Natural Killer Cells

NK cells are lymphoid cells with a central role in the innate response to viral infections and cancer cells, but recent data suggest that NK cells can also modulate the adaptive immune response involving DCs and T cells, either directly or indirectly (Moretta et al., 2008; Chiossone et al., 2018). Despite a deep search in literature, only a few papers concerning the role of MSC-EVs on NK cell modulation have been found. EVs derived from MSCs. EVs prevent proliferation and IL-2-induced activation of both CD56-dim and CD56-bright NK cells, and suppressed their cytotoxic degranulation *in vitro* (Fan et al., 2018). In a rat model of experimental autoimmune uveitis (EAU), MSC-EV administration reduces $CD161^+$ NK cell migration toward eye lesions, thus ameliorating EAU symptoms

(Bai et al., 2017). The protective and anti-inflammatory effects exerted by MSC-EVs have been also observed in a rat model of renal ischemic reperfusion injury (IRI) and in a renal allografts MHC-disparate rat model, by decreasing both NK cells infiltration and chemokines associated with NK cell recruitment (TLR-2 and CX3CL1) (Koch et al., 2015; Zou et al., 2016). All these immunosuppressive effects seem to be mediated by the expression of TGF-β on the EV surface, which induces TGF-β/Smad downstream pathway (Fan et al., 2018). Other molecules contained in MSC-EVs and associated with anti-inflammatory effects on NK cells are IL-10 and HLA-G (Kordelas et al., 2014). Finally, TNF-α- and IFN-γ-primed MSC-EVs reduce NK cell proliferation more effectively than resting MSC-EVs (Di Trapani et al., 2016).

MSC-EVs and Adaptive Immune System

B Cells

B cells are lymphoid cells involved in the humoral adaptive immunity through the secretion of antibodies and cytokines (Matsushita, 2019). Among the peripheral blood mononuclear cell (PBMC) subpopulations, B cells show the highest EV uptake (Di Trapani et al., 2016). MSC-EVs may induce in B cells the downregulation of 11 genes (including CCR3, CCR4, and CCL22) and the upregulation of 39 genes (including SerpinB2, PTGS2, and PGE2) involved in immune regulation (Khare et al., 2018). MSC-mediated inhibition of B cell proliferation is more evident following inflammatory priming (Di Trapani et al., 2016). Inflammatory priming induces the increase of miR-155 and miR-146 levels within MSC-EVs (Di Trapani et al., 2016). In particular, MSC-EVs induce the downregulation of PI3K/AKT signaling pathway components in B cells, inhibit B cell spreading, and reduce B cell viability via miR-155-5p (Adamo et al., 2019).

Another effect of MSC-EVs on B cells is preventing Ig secretion. MSC-EVs exert a dose-dependent inhibition of IgM, IgG, and IgA production coupled with suppression of B cell proliferation and maturation (Budoni et al., 2013). The reduction of IgG production was also observed by other authors reporting that both MSC-exosomes and microparticles may increase $CD19^+IL\text{-}10^+$ Breg-like population and inhibit plasmablast differentiation by transferring TGF-β, PEG2 and IL1RA (Cosenza et al., 2018). Moreover, MSC-EVs reduce $CD27^+CD19^+$ memory B cell maturation (Balbi et al., 2017). On the other hand, MSC-EVs may sustain, support and enhance the function of human IgG-secreting cells (Nguyen et al., 2018). Notably, MSC-EVs was not capable of significantly affect B cell activation in a strong reactive renal allotransplantation animal model; by contrast, MSC-EVs significantly increased the number of B cells infiltrating the transplanted kidney grafts (Koch et al., 2015). The partial immunomodulation of B cells by MSC soluble factors seems to be preferentially induced by the soluble protein-enriched fraction (PF) rather than by the entire EV-enriched fraction (Carreras-Planella et al., 2019).

T Cells

T cells are highly specialized lymphocytes that regulate several aspects of adaptive immunity, such as protection from pathogens, immune surveillance against tumors and alloreaction against non-self-tissues (Kumar et al., 2018). MSCs have a great impact on T cell functions and therefore potentially on the treatment of numerous T-cell mediated reactive conditions (Duffy et al., 2011).

An efficient approach to suppress T cell-mediated immune response is preventing T cell proliferation. Several studies reported that MSC-EVs exert this effect both *in vitro* and *in vivo* in several animal models, such as those reproducing myocardium infarction, experimental allergic asthma and renal IRI (Mokarizadeh et al., 2012; Kilpinen et al., 2013; Blazquez et al., 2014; Romani et al., 2015; Teng et al., 2015; de Castro et al., 2017; Monguió-Tortajada et al., 2017; Cosenza et al., 2018; van den Akker et al., 2018; Ji et al., 2019). The inhibition of T cell proliferation is associated with the reduction or absence of pro-inflammatory cytokines, such as IL-2, IL-6, TNF-α, and IFN-γ (Blazquez et al., 2014; Monguió-Tortajada et al., 2017). Nevertheless, MSC-EVs were capable of increasing T cell number in the graft of a rat renal transplant model for acute rejection associated with the reduction of TNF-α expression and no difference in IL-10 levels (Koch et al., 2015). The inhibition of T cell proliferation by human MSCs is mostly mediated by the upregulation of indoleamine 2,3-dioxygenase (IDO) (Chinnadurai et al., 2015; Wen et al., 2016); however, controversial results are found when T cells are treated with MSC-EVs. Some groups reported no significant changes in IDO expression (Del Fattore et al., 2015; Chen et al., 2016), whereas many authors found high concentrations of IDO inside MSC-EVs (Romani et al., 2015; Zhang et al., 2018b; Serejo et al., 2019). Other groups reported that MSC-EVs have no effect on T cell proliferation, but rather promote T cell apoptosis (Del Fattore et al., 2015; Chen et al., 2016). Conversely, another study reported that MSC-EVs do not alter T cell viability (Monguió-Tortajada et al., 2017). These different findings suggest that a thorough characterization of MSC-EV content and a standardization of the experimental methods are necessary to foresee the biological effects.

Both $CD4^+$ and $CD8^+$ T cell activation was suppressed by MSC-EVs. At molecular level, the suppression of T cell activation is independent from the antigen presentation due the lack of MHC class I and II as well as other costimulatory molecules on MSC-EV surface (Blazquez et al., 2014; Farinazzo et al., 2018; Dabrowska et al., 2019; Shao et al., 2020). In particular, MSCs constitutively lacking β2-microglobulin, a component of HLA-I involved in $CD8^+$ T cell-mediated immune rejection, and the corresponding EVs reduce more efficiently both fibrosis and inflammation in a myocardial infarction animal model compared to the wild-type forms (Shao et al., 2020). The authors reported a greater accumulation of miR-24 in EVs from MSCs constitutively lacking β2-microglobulin, which in turn reduces the expression of the apoptotic protein Bim (Shao et al., 2020). Additionally, MSC-EVs can block $CD4^+$ and $CD8^+$ T cell differentiation toward effector and memory cells, through a mechanism mediated by TGF-β signaling, respectively (Blazquez et al., 2014; Álvarez et al., 2018).

Modulation of Treg/Th17 and Th1/Th2 balance has been used to explain the regulatory properties of MSC-EVs on T cells. MSC-EVs may promote induction and expansion of Tregsin

association with high levels of IL-10 (Mokarizadeh et al., 2012; Kilpinen et al., 2013; Favaro et al., 2014; Del Fattore et al., 2015; Romani et al., 2015; Chen et al., 2016; Nojehdehi et al., 2018; Zhang et al., 2018b; Guo et al., 2019; Ji et al., 2019; Ma et al., 2019), particularly CTLA-4$^+$, CD4$^+$CD25$^+$Foxp3$^+$ and Tr1 Treg subpopulations (Chen et al., 2016; Cosenza et al., 2018). Other groups reported no significant changes in Treg number, regardless the higher IL-10 levels after MSC-EV treatment, thus questioning the involvement of Tregs in the upregulation of IL-10 expression by MSC-EVs (Hai et al., 2018). However, the promoting effects of MSC-EVs on Tregs could be partially mediated by their content in TGF-β signaling components (Song et al., 2020). Another possible molecular mechanism is the transfer of miR-1470 from MSC-EVs to CD4$^+$ T cells, thus upregulating P27KIP1 expression through c-Jun targeting (Zhuansun et al., 2019). Other miRNAs have been described in this phenomenon, such as miR155-5p, miR-let7b, and miR-let7d. The overexpression in MSC-EVs of miR-155, which targets Sirtuin-1, increases IL-10 and Foxp3 expression in T cells, thus preventing the production of IL-17 and RORC (Zheng et al., 2019). On the other hand, the increase of miR-let7b and miR-let7d may suppress cell proliferation and promote Treg functions, avoiding immune rejection (Wen et al., 2016). Moreover, MSC-EV-mediated proliferation and function of CD4$^+$CD25$^+$Foxp3$^+$ Tregs could involve APC-, but not CD4$^+$ T cell-dependent pathways (Du et al., 2018). Regardless the mechanism mainly involved, other *in vivo* models, such as experimental type-1 autoimmune diabetes in T1D mice, clearly showed that the induction of Tregs by MSC-EVs can ameliorate histological signs, thus favoring the regeneration of tissues, i.e., pancreatic islets (Nojehdehi et al., 2018).

Concerning other T-cell subsets, there are only a few works so far. For instance, MSC-EVs may prevent Th17 cell development and IL-17 production (Favaro et al., 2014; Chen et al., 2016; Bai et al., 2017; Shigemoto-Kuroda et al., 2017; Hai et al., 2018; Ji et al., 2019; Ma et al., 2019). MSC-EVs may also inhibit Th17 cell differentiation in ulcerative colitis rat models by increasing histone H3K27me3 methylation and inhibiting its demethylation, thus suggesting that H3K27me3 may be an important target in inflammatory diseases (Chen et al., 2020). Moreover, MSC-EVs can directly prevent Th1 development by promoting Th1 shift toward Th2 cells (Chen et al., 2016; Bai et al., 2017; Shigemoto-Kuroda et al., 2017; Guo et al., 2019) as well as inhibit T follicular helper cells (Hai et al., 2018). Nevertheless, MSC-EVs can also promote autoreactive, IFN-γ-secreting memory Th1 cells by functioning in NOD mice as self-antigen carrier and trigger for autoimmunity (Rahman et al., 2014). In addition, the effect of MSC-EVs on natural killer-T (NK-T) cells has been recently described in a rat model of hepatocellular carcinoma; following EV administration, higher percentages of circulating and intratumoral NK-T cells as well as tumors of smaller size and less aggressive were observed as compared to untreated rats (Ko et al., 2015).

Different mechanisms and factors have been described in the immunomodulatory effect of MSC-EVs toward T-cells. The broad and pleiomorphic activity of MSC-EVs reflects their influence on different signaling pathways of T-cells and microenvironmental cells, such as JAK/STAT or NF-kB (Guo et al., 2019). For instance, MSC-EVs can inhibit T-cell infiltration in the injury site of several diseases as well as the production of several chemokines, such as CCL2, CCL5, CCL21, CXCL1, MIP-1α, MIP-3α, and integrin-dependent chemokines) (Cruz et al., 2015; Bai et al., 2017; Shigemoto-Kuroda et al., 2017; Farinazzo et al., 2018; Hai et al., 2018; Dabrowska et al., 2019) and inflammatory molecules, such as IL-1α, IL-1β, IL-2, IL-5, IL-12, and IL-17 (Favaro et al., 2014; Chen et al., 2016; de Castro et al., 2017; Shigemoto-Kuroda et al., 2017; Hai et al., 2018; Nojehdehi et al., 2018; Dabrowska et al., 2019; Guo et al., 2019; Ji et al., 2019; Ma et al., 2019). By contrast, anti-inflammatory molecules can be induced by MSC-EVs, such as IL-10, TGF-β, and PGE2 (Mokarizadeh et al., 2012; Favaro et al., 2014; Del Fattore et al., 2015; Chen et al., 2016; Nojehdehi et al., 2018; Guo et al., 2019; Ji et al., 2019; Ma et al., 2019). Other factors, such as IL-4, IL-6, IFN-γ, and TNF-α, seem to be variably modulated by MSC-EV (Rahman et al., 2014; de Castro et al., 2017; Shigemoto-Kuroda et al., 2017; Hai et al., 2018; Nojehdehi et al., 2018).

Inflammatory priming may enhance the immunomodulatory properties of MSC-EVs. For instance, inflammatory IL-1β-priming MSC upregulates PD-L1 and TGF-β expression in EVs, leading to a Treg increment in a mouse model of autoimmune encephalomyelitis (Mokarizadeh et al., 2012). A greater accumulation of TGF-β was also reported in IFN-γ-primed MSC-EVs, which also showed low levels of Galectin-1 and IDO, compared to resting MSC-EVs, leading to a suppression of Treg expansion (Serejo et al., 2019). Compared to resting MSC-EVs, TNF-α, and IFN-γ-primed MSC-EVs reduced more the TNF-α and IFN-γ secretion from splenocyte previously activated with lipopolysaccharides and concanavalin A to preferentially stimulate either myeloid cells or T cells, respectively (Harting et al., 2018). According to the authors, the best efficiency of inflammatory priming was probably due to the higher concentration of COX2 and PGE2 in primed MSC-EVs (Harting et al., 2018). Intriguingly, EVs from MSCs pretreated with a combination of anti- and pro-inflammatory cytokines (TGF-β and IFN-γ, respectively) promote Treg expansion more efficiently than MSC-EVs pretreated with TGF-β or IFN-γ only and display higher levels of IDO, IL-10, and IFN-γ (Zhang et al., 2018b). Nevertheless, the promoting effect of inflammatory priming was not confirmed by other authors (Kilpinen et al., 2013; Cosenza et al., 2018), who either found a major effect of resting MSC-EVs, or a negligible effect on T cell proliferation of both resting and primed (TNF-α and IFN-γ) MSC-EVs (Kilpinen et al., 2013; Di Trapani et al., 2016; Cosenza et al., 2018).

Altogether, these data give an idea about the complexity of the interactions and effects that can be mediated by MSC-EVs in physiological and reactive conditions, depending on microenvironmental factors, activating stimuli, effector cell subsets and cellular cross-talk. This scenery becomes even more complex when MSC-EVs are administered as cell-free therapeutic approaches in autoimmune or inflammatory conditions.

MSC-EV-Based Immunotherapy

MSC systemic administration, which must follow Good Manufacturing Practice (GMP) rules, is not associated to

a significant evidence of cell engraftment even in presence of clinical benefit, due to the entrapment of MSCs in the microvasculature of filter organs, such as lungs (Moll et al., 2016; Salvadori et al., 2019). Other biology aspects can interfere with therapeutic efficacy of MSCs. For instance, the quality and the integrity of MSC preparations depends on the isolation, culture, and cryopreservation methods (Moll et al., 2016; Dufrane, 2017; Mastrolia et al., 2019). Although autologous MSCs would be the best choice for MSC therapy, they showed some limitations: patients' age as well as their genetic traits and medical conditions could reduce the proliferation rate and therapeutic features of MSCs (Pachón-Peña et al., 2016; Dufrane, 2017). Limitations have been also observed in allogeneic MSC transplantation. Indeed, despite MSC have been always considered characterized by a low immunogenic potential, recent studies demonstrated that MSCs may elicit anti-donor immune response (Ankrum et al., 2014; Lohan et al., 2017). Therefore, in order to switch toward a cell-free approach, many groups began to study the immunomodulatory effects of MSC-EVs administered *in vivo*. One of the first clinical setting in which employing MSC-EVs was acute GvHD, the main complication of allogeneic hematopoietic stem cell transplantation (HSCT) (Ferrara et al., 2009; Szyska and Na, 2016; Zeiser and Blazar, 2017). Acute GvHD (aGvHD) occurs within 40 days after HSCT transplantation, as a consequence of interactions between mature donor T cells and host and donor APCs, mounting a strong immune response that eventually lead to host tissue damage (Tyndall and Dazzi, 2008; Zeiser and Blazar, 2017). On the other hand, chronic GvHD (cGvHD) can arise de novo or from aGvHD and is a more complex disease involving not only mature donor T cells, but also auto/alloreactive B cells escaping negative selection (Toubai et al., 2008; Zeiser and Blazar, 2017; Hill et al., 2018). Despite several prophylactic and therapeutic strategies have been developed, the mortality rate of refractory aGvHD is still 70–80%, mostly due to severe secondary infectious complications (Jamil and Mineishi, 2015; Hamilton, 2018).

MSCs initially represented an interesting candidate for cellular therapy to improve HSCT engraftment, prevent graft failure and treat refractory aGvHD. Despite several preclinical and clinical studies showing clinical and survival improvement in MSC-treated patients compared to controls, a significant number of clinical trials failed, especially in adults, probably due to the lack of appropriate knowledge of the mechanisms of action when MSCs are administered *in vivo* (Elgaz et al., 2019; Cheung et al., 2020). For this reason, several groups started to investigate the effectiveness of MSC-EVs in aGvHD *in vivo* models and patients. MSC-EVs may prevent aGvHD onset, attenuate symptoms, and prolong animal survival through several mechanisms. For instance, MSC-EVs is capable of reducing $CD8^+$ T cell number, leading to the increase of $CD4^+/CD8^+$ T cell ratio; in addition, they block $CD4^+$ T cell migration and activation inside target organs, promote Treg expansion, downregulate IL-2, CCR6, TNF-α, and IFN-γ expression while increasing IL-10, reduce Th17 cell recruitment while lowering RORγτ, STAT3, IL-17, IL-21, IL-22 expression (Wang et al., 2016; Fujii et al., 2018; Lai et al., 2018; Zhang et al., 2018a; Dal Collo et al., 2020). Other potential MSC-EV immunomodulatory mechanisms on T cells involve miR-223 and the adenosine metabolism. miR-223, which is highly expressed in EVs from umbilical cord, is capable of inhibiting allogenic T cell migration and extravasation by targeting ICAM-1, thus leading to a reduction of pro-inflammatory factors and GvHD symptoms (Liu et al., 2020). Regarding adenosine metabolism, it has been observed in a humanized GvHD mouse model that MSC-EVs can transfer CD73 to CD39 enzyme on the surface of tissue-infiltrating Th1 cells, thus inducing a significant production of adenosine that eventually reduces CD39 expression, enhances apoptosis of adenosine A2A receptor-expressing Th1 cells, and downregulates IFN-γ and TNF-α expression, without inducing Tregs (Amarnath et al., 2015). The involvement of adenosine metabolism in T cell modulation was also confirmed by other groups (Kerkelä et al., 2016; Crain et al., 2018). Interestingly, the anti-GvHD function is restricted to MSC-EVs, as human dermal fibroblast-derived EVs are devoid of these effects (Fujii et al., 2018). MSC-EV treatment was also tested in a therapy-refractory GvHD patient, who showed GvHD clinical symptoms improvement and remained stable for several months (Kordelas et al., 2014): MSC-EV preparations contained high concentrations of IL-10, TGF-β, and HLA-G that paralleled with the decrease in the number of both PBMCs releasing IL-1β, TNF-α, and IFN-γ and stimulated NK cells releasing TNFα- or IFN-γ (Kordelas et al., 2014).

Unfortunately, not all EV preparations from MSCs are functionally equivalent (Madel et al., 2019). Therefore, it is necessary to characterize the functional activity of MSC-EV preparations and to identify predictive tests that may foresee the clinical benefit. Kordelas et al. (2019) proposed an *in vitro* assay to monitor the impact of different EV preparations from human donor bone marrow MSCs (BM-MSCs)-MSCs on T cell differentiation and corresponding cytokine production. Recently, a functional *in vitro* assay was suggested to assess the MSC-EV therapeutic dose (EV-TD) *in vivo* in a mouse model of aGvHD; EV-TD, associated with the improvement of mouse overall survival, corresponded to 10-fold the EV immunomodulatory functional unit (EV-IFU), i.e. the lowest concentration *in vitro* of resting MSC-EV-pool leading to at least threefold increase of Tregs compared to control (Dal Collo et al., 2020). Nevertheless, all these assays need to be validated in a large cohort of patients before being accepted as predictive methods of MSC-EV therapeutic efficacy.

Other clinical studies employing MSC-EVs as treatment of many diseases with inflammatory phenomena are reported on clinicaltrials.gov. According to our search, using the terms "mesenchymal extracellular vesicles" and "stromal extracellular vesicles," only three clinical studies have been registered concerning bronchopulmonary dysplasia (NCT03857841)[1], osteoarthritis (NCT04223622)[2], and dystrophic epidermolysis bullosa (NCT04173650)[3]. In particular, NCT03857841 study will employ UNEX-42, a preparation of EVs secreted from human BM-MSCs suspended in phosphate-buffered saline; NCT04223622 study will use the entire secretome or EVs derived

[1]https://clinicaltrials.gov/ct2/show/NCT03857841

[2]https://clinicaltrials.gov/ct2/show/study/NCT04223622

[3]https://clinicaltrials.gov/ct2/show/study/NCT04173650

from adipogenic MSCs; and NCT04173650 study will employ AGLE-102, an allogeneic derived EV product derived from normal donor MSCs. However, all studies are currently ongoing and no clear-cut results have been reported so far.

CONCLUSION

Immunomodulatory capacity of MSCs is associated, at least in part, with the release of EVs. The ability of MSC-EVs to affect immune response, promoting immunotolerance in tissue microenvironment, opens new cues on intercellular communication through soluble factors and makes MSC-EVs a new promising therapeutic strategy for the treatment of many inflammatory disorders. Compared to cell therapy, EV treatment offers a number of advantages in terms of higher distribution in target organs, lower immunogenicity and tumorigenicity as well as easier handling and preparation procedures. Unfortunately, MSC-EVs can have variable biological effects on the same effector cell type depending on different factors, such as the quality of primary cells, MSC source, culture conditions, preconditioning with inflammatory cytokines, cryopreservation methods, purification and quantification protocols, etc. (Théry et al., 2018). These premises, together with the lack of standardized approaches, specific dosing and defined quality controls for clinical use, require further investigations before transferring EV-based treatments from bench to bedside.

AUTHOR CONTRIBUTIONS

RB wrote the manuscript and prepared the figure. PT and IT critically revised the manuscript. MK planned and revised the final version of the manuscript. All authors approved the submitted version of the manuscript.

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Amnion-Derived Mesenchymal Stem Cell Exosomes-Mediated Autophagy Promotes the Survival of Trophoblasts Under Hypoxia Through mTOR Pathway by the Downregulation of EZH2

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Human amnion-derived mesenchymal stem cells (AD-MSCs) have been reported as a promising effective treatment to repair tissue. Trophoblast dysfunction during pregnancy is significantly involved in the pathogenesis of preeclampsia (PE). To understand how AD-MSCs regulated trophoblast function, we treated trophoblasts with AD-MSC-derived exosomes under hypoxic conditions. The treatment markedly enhanced the trophoblast proliferation and autophagy. Furthermore, significant decrease of EZH2 levels and inactivation of mTOR signaling were observed in AD-MSC exosomes-treated trophoblasts. Consistent with these findings, overexpression of EZH2 activated the mTOR signaling in trophoblasts, and reduced the autophagy and survival of trophoblasts, even in the presence of AD-MSC-derived exosomes. In addition, EZH2 inhibition exhibited the same trophoblast autophagy-promoting effect as induced by AD-MSC-derived exosomes, also accompanied by the inactivation of mTOR signaling. Importantly, when EZH2 was overexpressed in trophoblasts treated with PQR620, a specific mTOR signaling inhibitor, the autophagy and proliferation in trophoblasts were decreased. Studies on human placental explants also confirmed our findings by showing that the expression levels of EZH2 and mTOR were decreased while the autophagy-associated protein level was increased by AD-MSC-derived exosome treatment. In summary, our results suggest that EZH2-dependent mTOR signaling inactivation mediated by AD-MSC-derived exosomes is a prerequisite for autophagy augmentation in hypoxic trophoblasts.

Keywords: chorionic villous-derived mesenchymal stem cells, trophoblasts, autophagy, EZH2, mTOR signaling

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INTRODUCTION

Preeclampsia (PE) is a hypertensive disorder during pregnancy, and is associated with substantial maternal and perinatal complications (Hladunewich et al., 2007; Rana et al., 2019). It is deemed to be one of the main causes of fetal and maternal death and morbidity across the world, and is reported to have a 2–8% incidence among pregnancies, particularly in the developing countries (Tranquilli et al., 2012). Despite the considerable efforts on its study, the etiology of PE has not yet been clear. At present, uteroplacental malperfusion caused by the trophoblast invasion failure and transformation of spiral arteries is considered as an important cause for this disorder (Ridder et al., 2019). In addition, aberrant placental implantation can cause increased oxidative stress and hypoxia, which leads to inflammation and antiangiogenic protein release (Mol et al., 2016). Ischemia and hypoxia in trophoblasts are important pathological manifestations of PE.

Autophagy is a mechanism that maintains homeostasis through degradation of malfunctioned organelles, redundant molecules and invasive pathogens by lysosomes (Kang et al., 2020). Autophagy also plays important roles in intracellular signaling pathways regulating cell proliferation, differentiation, and replicative senescence (Mo et al., 2020). Under stress conditions such as malnourishment, hypoxia and starvation, autophagy would be activated to provide nutrients and energy to the cells (Mizushima and Komatsu, 2011; Ho et al., 2017). Due to the hypoxic and ischemic responses associated with PE, placental trophoblasts in individuals with PE are more reliant on autophagy for survival than normal cells (Nakashima et al., 2019a,b). Autophagy is crucial in many tissues, including that in placenta (Menikdiwela et al., 2020). It protects syncytiotrophoblasts from apoptosis, infection, and inflammation in the human placenta (Zenclussen et al., 2017; Zhang et al., 2019), but many questions remain regarding the exact etiology and precise pathogenic mechanisms in the autophagic flux.

EZH2 is a bona fide histone methyltransferase, methylating histone 3 at lysine 27 (H3K27) and leading to a selective suppression of transcription by changing the chromatin conformation (Aalia Batool and Liu, 2019). Due to its histone methyltransferase activity, epigenetic mechanisms are often cited in explaining the biological consequences following its suppression or overexpression (Huang et al., 2014; Mu et al., 2017). The role of EZH2 in cancer progression and malignancy has been extensively studied in the last decade (Liu et al., 2018; Emran et al., 2019). Moreover, growing evidences demonstrate that downregulation of EZH2 was essential for inducing autophagy and apoptosis in many types of cancer cells (Xue et al., 2019; Liu et al., 2020). However, the exact role EZH2 plays in trophoblastic autophagy has not been fully elucidated.

Mesenchymal stem cells (MSCs) are versatile cells that can be differentiated into various specialized cells including osteoblasts, scleroblasts, chondrocytes, and adipocytes (Ridge et al., 2017). AD-MSCs have similar phenotypic and functional characteristics to other kinds of MSC (Umezawa et al., 2019). An extra beneficial characteristic of MSC is its capability to attenuate inflammation and tissue ischemia (Bianco et al., 2008; Mazini et al., 2020). These characteristics could be favorable for therapeutic placental villi remodeling and for promoting placental development. Many studies have suggested that AD-MSCs can promote angiogenesis through paracrine effects and potentially participate in placental pathologies in the vascular system, including PE as well as fetal growth restriction (MacDonald and Barrett, 2019). However, currently there is no information on the roles of AD-MSCs in the autophagic capacity of extravillous trophoblasts (EVTs).

In this study, the effects of AD-MSC-derived exosomes on the proliferative capacities of the trophoblast cell lines JEG-3 and HTR-8 were studied. In addition to increasing the proliferative capacities, AD-MSC-derived exosomes significantly enhanced autophagy in the trophoblasts under hypoxic conditions. The transcriptome analysis showed considerable downregulation of the enhancer of zeste 2 polycomb repressive complex 2 subunit (EZH2) and mTOR signaling pathway in trophoblasts treated with treated with AD-MSC-derived exosomes; mTOR was putatively recognized as an upstream inhibitor of autophagy under hypoxic conditions.

MATERIALS AND METHODS

Cell Culture

Placentas were obtained from full-term births after a cesarean section (n = 3) with parental permission. Every procedure was conducted in accordance with the ethical protocols of The Affiliated Hospital of Qingdao University, China. Amnions were separated from term placentas. Primary AD-MSCs were extracted from term amnion. AD-MSCs were isolated, cultured and characterized as published (Konig et al., 2012; König et al., 2015). All AD-MSCs were used at passages 3–5 in this study. The isolated cells were plated on culture plates in stem cell culture medium (SCCM), which contained Stem Cell Basic Medium (Dakewe Biotech Co., Guangzhou, China) and 5% UltraGRO^{TM} (Helios, United States). An incubator with a temperature of 37°C and 5% CO_2 was used to culture The primary cells were cultured in an incubator at 37°C in an atmosphere of 5% CO_2.

The JEG-3 and HTR-8 cells obtained from the Type Culture Collection of China Centre were subjected to culture and then used for experiments. DMEM/F12 containing 10% FBS was used to culture all three trophoblast cell lines in an incubator at 37°C and in an atmosphere of 5% CO_2. The medium was changed when the confluency reached 50%. The cells were subjected to incubation for a set of time at 37°C, and the humidified atmosphere of the incubator contained 93% N_2, 5% CO_2, and 2% O_2 (Invivo2 Hypoxia Workstation, Ruskinn Technology, Leeds, West Yorkshire, United Kingdom). For each experiment, the cells were subjected to culture in triplicate.

AD-MSC Identification

Flow cytometry (with antibodies obtained from eBioscience, San Diego, CA, United States, including CD34, CD105, CD73, CD90, CD44, CD45, IG1, and HLA-DR) was used to examine the expression of cell markers in AD-MSCs (passage 3); these markers included positive markers (CD44, CD73, CD90,

and CD105) and negative markers (CD34, CD45, CD146, IG1, and HLA-DR).

Moreover, AD-MSCs from normal placentas or placentas with severe PE could differentiate into osteoblasts as well as adipocytes; therefore, we assessed their differentiation capability. AD-MSCs cultured in 6-well plates were grown to approximately 70–80% confluency. Then, the AD-MSCs were cultured in differentiation medium (osteogenic or adipogenic) (Gibco, Carlsbad, CA, United States) for 3 weeks. Alizarin red S was used to stain the AD-MSCs to verify osteoblast differentiation; for adipocyte differentiation, oil red O was selected.

Exosome Isolation

Exosomes were obtained from epidural AD-MSC supernatants by differential centrifugation. The medium was discarded when AD-MSCs reached 70% confluency. Then, the cells were cultured in serum-free DMEM/F12 for another 24 h. The supernatants were collected and then cleared by sequential centrifugation at 15,000 × *g* for 30 min or 3,000 × *g* for 30 min. The supernatants were ultracentrifuged at 120,000 × *g* for 2 h after being filtrated through 0.22-mm filters (Millipore, Billerica, MA, United States). The exosomes were rinsed by sterile PBS and collected several times. The exosome concentrations were determined with a Pierce BCA protein assay kit (Thermo Fisher Scientific).

Electron Microscopy

In approximately 10 min, almost 50 μl of prepared exosomes were adsorbed and placed onto formvar carbon-coated 300-mesh copper grids. Then, the adsorbed exosomes were dried at room temperature for 30 min and negatively dyed with 3% phosphotungstic acid. Later, by using a transmission electron microscope (Olympus Software Imaging Solutions) at 120.0 kV, the exosomes were examined. Moreover, a digital camera was used to capture images of the exosomes.

Antibodies and Reagents

We purchased anti-microtubule-associated protein LC3, P62, CD63, and BECN1 (beclin1, an autophagosome initiator) antibodies from R&D Systems (Minneapolis, MI, United States). Cell Signaling Technology (Danvers, MA, United States) provided the following antibodies: anti-EZH2 (#5246), anti-mTOR (#2983), anti-p-mTOR (#5536), anti-S6K1 (#2708), anti-p-S6K1 (#9204), anti-TSG101 (#28405), and anti-Ki67 (#9449). GSK126 (EZH2inhibitor) (10 μM), PQR620 (50 nM) and bafilomycin A1 (Baf A1, an autophagosome-lysosome fusion inhibitor) (100 nM) were purchased from MedChem Express (Monmouth Junction, NJ, United States). EZH2 plasmids were purchased from Shanghai Genechem Co., Ltd.

Quantitative Real-Time PCR

AD-MSC-derived exosomes (10 μg/ml) was used to treat JEG-3, and HTR-8 cells for 24 h. Then, we isolated the total RNA from the trophoblasts with TRIzol Reagent (Takara, Japan). After that, a reverse transcription kit (Invitrogen) was used to synthesize complementary DNA. Master Mix (Thermo Fisher Scientific) and Gene-specific TaqMan probes (Applied Biosystems) were used to carry out quantitative real-time PCR (RT-PCR) according to the manufacturer's instructions. The expression of each target gene was normalized to GAPDH expression. We used TaqMan probes for EZH2 (Hs00544830_m1), mTOR (Hs00234508_m1), S6K1 (Hs00356367_m1), and GAPDH (Hs02786624_g1), and conducted three separate reactions for each marker.

RNA Interference

Short interfering RNA (siRNA) oligonucleotide duplexes targeting EZH2 used in this study were synthesized and purified by RiboBio (Ribobio Co., Guangzhou, China). The sequences are as follows: siEZH2 #1: 5′-GCUGGAAUCAAAGGAUACA-3′; siEZH2 #2: 5′-GCGTTTCTTGTATCGGGAAAT-3′. A nonsense siRNA with no homology to the known genes in human cells was used as negative control: 5′—UUC UCC GAA CGUGUC ACG UTT—3′. Transfections of siRNA in trophoblasts were performed by using Lipofectamine 2,000 (Invitrogen, Carlsbad, CA, United States) according to the manufacturer's instructions, and the knockdown efficiency was verified 48 h after transfection. All the siRNAs were used at a final concentration of 100 nM.

Construction of Vectors

To make EZH2 constructs and its mutants, the human EZH2 gene was amplified by PCR and cloned into the SgfI/MluI sites of the pCMV6-Entry vector (Life Technologies).

Cell Proliferation Analysis

We added trophoblasts to ninety-six-well plates (density: 5,000 cells per well), cultured these cells, and measured trophoblast proliferation daily via CCK-8 assays (Thermo Fisher Scientific, Waltham, MA, United States). We added CCK-8 reagent to each well, and cultured the trophoblasts for another 1.5 h. Then, colorimetric assays were performed by measuring the absorbance [optical density (OD) value] of each well in a microplate reader (wavelength: 450 nm). The growth curves were ascertained in three separate experiments.

EdU Assay

According to the manufacturer's instructions (Guangzhou RiboBio, Guangzhou, China), 5-Ethynyl-2'-deoxyuridine (EdU) assays were conducted by using a Cell-Light EdU *in vitro* flow cytometry kit. In brief, in a 6-well plate cells were cultured overnight, with a 20-min incubation with EdU followed. Then, they were fixed with 70% ethanol at −20°C overnight and washed twice with PBS. Later, the cells were stained with a FITC-conjugated secondary antibody at ambient temperature for 1 h and denatured in 2 N HCl for 45 min. Moreover, with 40 g/ml RNase A and 200 g/ml PI, the cells were incubated for 30 min and finally analyzed by flow cytometry.

Western Blotting

Trophoblasts were lysed on ice for 12 min with RIPA buffer (Sigma, St. Louis, MO, United States). After centrifugation at 12,000 × *g*, the cell lysates were treated with LDS sample buffer. SDS-PAGE was used to separate the protein mixtures, which were then electro-transferred to a polyvinylidene fluoride (PVDF)

membrane (Bio-Rad, Hercules, CA, United States). Next, 5% skim milk was used to block the membrane.

Subsequently, primary rabbit monoclonal antibodies against human LC3, BECN1, P62, EZH2, mTOR, p-mTOR, S6K1, and p-S6K1 (1:1,000 dilution) or β-actin (same dilution; Proteintech, Chicago, IL, United States) were incubated with the blocked membranes. Then, secondary antibodies were incubated with the membranes (1:1,000; CST, Danvers, MA, United States). The protein-antibody complexes were detected and quantified by using a chemiluminescence detection system (Bio-Rad, Hercules, CA, United States).

Placental Explant Culture

All placentas were collected after operation, treated within 30 min and closely examined for any visible abnormalities. After thorough rinsing with PBS 3 times to remove the maternal blood, the placental villous tissues were chopped into 8-mm^3 pieces (2 mm × 2 mm × 2 mm). DMEM/F12 (4 ml per well) with 1% penicillin/streptomycin and amphotericin B (Gibco, Carlsbad, CA, United States) was used to culture the placental explants in six-well dishes (Corning) in a hypoxic incubator for 48 h at 37°C in an atmosphere of 2% oxygen. After AD-MSC-derived exosomes treatment for 24 h, PBS was used to rinse the explants; after that, they were frozen in liquid nitrogen.

Immunohistochemistry

Paraformaldehyde (4%) was used to fix the human term placental explants for 60 min. We embedded the tissues in paraffin, sliced them into 4-μm sections, and deparaffinized them. Then, the slides were boiled in 6.0 pH sodium citrate buffer (10 mM) for 7 min at 120°C for antigen retrieval. Hydrogen peroxide was used to block endogenous peroxidase for 10 min. We subsequently washed the slides three times for 5 min each with TBS (containing 0.05% Tween 20) (TBS/T; Merck; Darmstadt, Germany); later, these slides were incubated with monoclonal anti-EZH2 antibodies (1:200) for 12 h at 4°C. Diluted biotin-labeled secondary antibodies were incubated with these sections for 20 min at 37°C. We visualized the target proteins via fresh DAB solution and used hematoxylin as a tissue counterstain. Using an optical microscope (Olympus FV500, Tokyo, Japan), the expression of the target proteins was assessed by two observers independently. The staining area and intensity in five different random regions (200× magnification) were analyzed with Image-Pro Plus 5.1 to assess the protein expression levels.

Immunofluorescence

Trophoblasts were cultured with or without purified AD-MSC-derived exosomes for 24 h (10 μg/ml). Then, all the cells were gathered and separated, and later for 60 min, they were fixed with 4% paraformaldehyde. The fixed cells were cut into 4-μm sections and embedded in paraffin. Later, they were washed in PBS for three times and blocked with 10% goat serum for 1 h. After that in 0.2% Triton X-100 the sections were washed twice. Next, they were incubated with primary antibodies (anti-LC3-II and anti-tubulin were purchased from Abcam, Cambridge, MA, United States), secondary antibodies (Invitrogen) and DAPI (Guangzhou RiboBio, Guangzhou, China). Using a fluorescence microscope, images were captured.

Statistical Analysis

One-way ANOVA or two-tailed Student's *t*-test was adopted to carry out statistical analysis; the data are reported as the mean ± standard deviation (SD) from more than three experiments performed independently. If the *P* value was less than 0.05, it indicated a statically significant difference.

RESULTS

Proliferation and Autophagy Were Promoted in Trophoblasts by AD-MSCs Under Hypoxic Conditions

First, we isolated AD-MSCs from healthy placentas, and identified their multidirectional differentiation ability and surface marker expression (**Supplementary Figures 1A,B**). Then, we identified the exosomes isolated from AD-MSCs and observed the exosomes endocytosis in trophoblasts (**Supplementary Figure 1C**). Next, we used the CCK-8 assays, EdU assays, immunofluorescence and Western blotting to examine the proliferation and autophagy of trophoblasts treated with or without AD-MSC-derived exosomes under hypoxic conditions (**Figures 1A–D**). The AD-MSC-derived exosome-treated trophoblasts exhibited significantly higher proliferation rates than the untreated cells under hypoxic conditions (**Figures 1A,B**, all $P < 0.001$). To further verify the autophagy-promoting effect of AD-MSC-derived exosomes on trophoblasts, we examined the LC3-II/LC3-I ratio, BECN1 and P62 levels in trophoblasts treated with AD-MSC-derived exosomes by western blotting. The AD-MSC-derived exosome-treated trophoblasts exhibited significantly higher levels of LC3 and BECN1 than the untreated cells (all $P < 0.05$), and the P62 levels of the two trophoblast cell lines was decreased after treatment with AD-MSC-derived exosomes (**Figure 1C**). According to the immunofluorescence assay results, AD-MSC-derived exosomes increased the staining intensity and area of punctate LC3-II in trophoblasts after 24 h of exposure to the hypoxic culture system (**Figure 1D**). These results indicated that the AD-MSCs promoted trophoblast proliferation and autophagy under hypoxic conditions by secreting exosomes.

The EZH2 Expression Was Underregulated in Trophoblasts by AD-MSC-Derived Exosome Treatment Under Hypoxic Conditions, Also Accompanied by the Inhibition of mTOR Pathway

To better understand the changes in trophoblasts that occurred after AD-MSC-derived exosome treatment, we compared the EZH2 levels in the two trophoblast cell

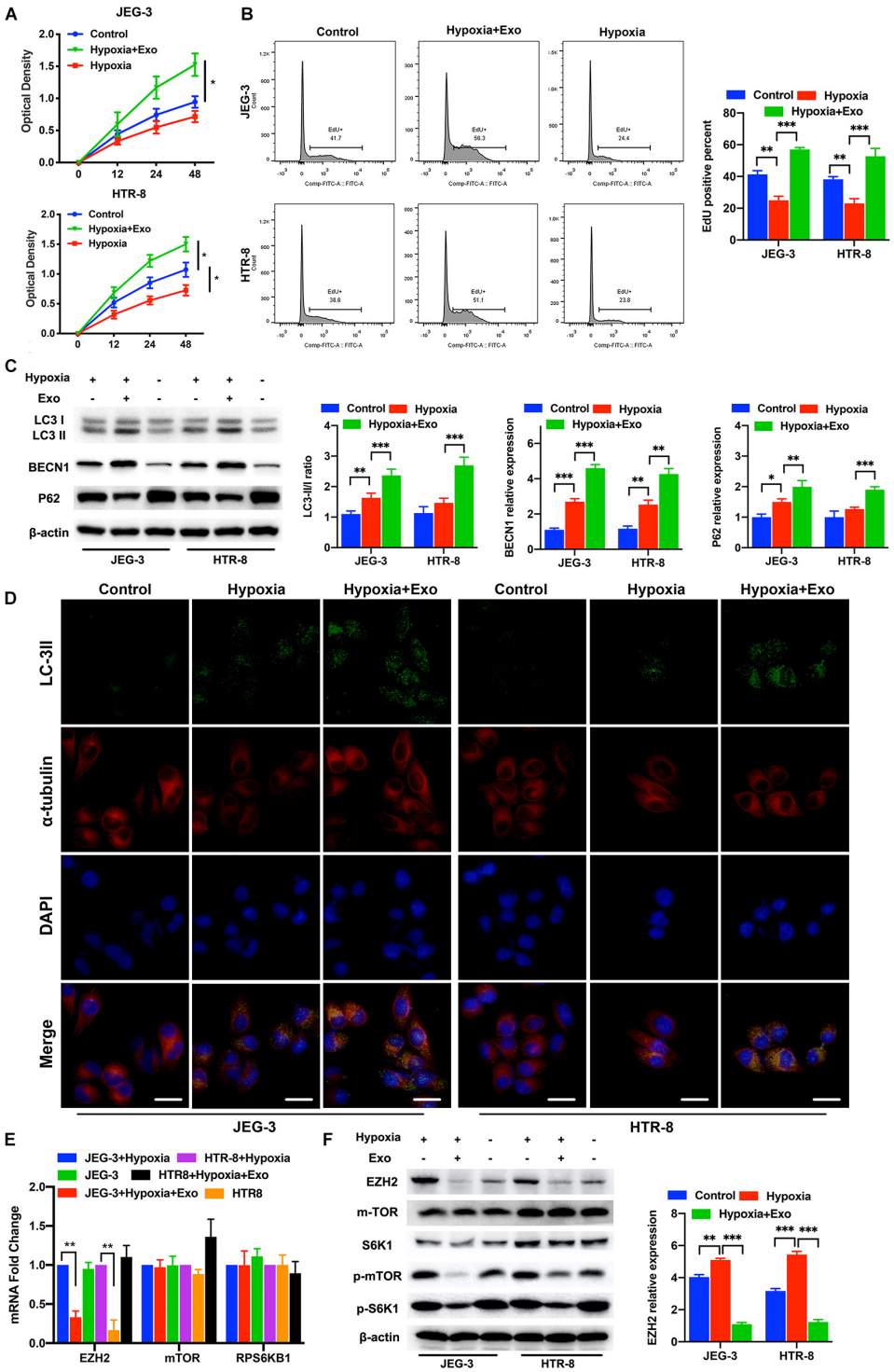

FIGURE 1 | AD-MSC-derived exosomes promote the autophagy and proliferation of trophoblasts in hypoxia condition. **(A)** Representative CCK-8 assay results for JEG-3 and HTR-8 cells are shown. Trophoblast cells were treated with AD-MSC exosomes under hypoxic conditions. **(B)** Representative EdU assay results for trophoblasts are shown. The histogram of EdU positive trophoblasts that treated with AD-MSC exosomes under hypoxic conditions was shown. **(C)** Whole cell lysates from trophoblast cells were subjected to western blotting to analyze and quantificate LC3, BECN1 and p62 levels. β-actin was included as a loading control. **(D)** Immunofluorescence analysis showed autophagosomes in the cytoplasm of trophoblast cells treated with AD-MSC exosomes under hypoxic conditions (scale bar, 25 μm). **(E)** Significant decreases in EZH2, mTOR and S6K1 mRNA levels were found in JEG-3 and HTR-8 cells treated with AD-MSC exosomes by qRT-PCR. **(F)** Whole cell lysates from trophoblast cells were subjected to western blotting to analyze EZH2, mTOR, S6K1, p-mTOR and p-S6K1 levels and quantificate EZH2 expression. β-actin was included as a loading control. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

lines cultured for 48 h with or without AD-MSC-derived exosomes under hypoxic conditions. The mRNA levels of EZH2 significantly decreased in trophoblasts treated with AD-MSC-derived exosomes (**Figure 1E**). Many researches have revealed that the mTOR pathway plays a vital role in intracellular metabolism regulation and could regulated by EZH2, suggesting that it is involved in regulating autophagy (Wei et al., 2016; Hu, 2019). Therefore, we examined whether the mTOR pathway could be regulated by the AD-MSC-derived exosomes. Our results revealed that AD-MSC-derived exosome-treated trophoblasts (48 h treatment) exhibited lower mTOR and RPS6KB1 mRNA levels than untreated trophoblasts (**Figure 1E**). In consistence with the data of mRNA levels, the EZH2, p-mTOR and p-S6K1 protein levels were significantly decreased in AD-MSC-derived exosome-treated trophoblasts compared to the control trophoblasts (**Figure 1F** and **Supplementary Figure 2B**). Our results suggested that the mTOR signaling pathway could be inhibited by AD-MSC-derived exosome treatment in trophoblasts under hypoxic conditions, which is also accompanied by decreased EZH2 expression.

The Downregulation of EZH2 Induced the Increase of Trophoblast Autophagy and mTOR Pathway Inhibition Under Hypoxic Conditions

After confirming that AD-MSC-derived exosomes activated autophagy and inhibited the EZH2 expression in trophoblasts, we investigated whether EZH2 could regulate the trophoblast autophagy. Trophoblasts were treated with the specific EZH2 inhibitor GSK126 and autophagy inhibitor Baf A1. The results confirmed the inhibiting effective concentration of GSK126 on the H3k27me3 levels in trophoblasts (**Supplementary Figure 1D**), and 10 μM GSK126 was used in further study. LC3-II/ LC3-I ratio and BECN1 levels in trophoblasts were significantly increased, P62 levels were decreased in the presence of GSK126 (10 μM), whether with or without AD-MSC-derived exosome. Meanwhile Baf A1 treatment increased LC3-II levels because of blocking autophagic flux (**Figures 2A–C**). In consistence with the data of LC3-II/ LC3-I ratio in Western blotting, GSK126 and ADSCs exosomes promote the LC3-II levels in trophoblasts by Immunofluorescence assays (**Supplementary Figure 3A**). In addition, we discovered that p-mTOR, p-RPS6KB1 and P62 protein levels decreased; and the LC3-II/LC3-I ratio and BECN1 level increased in the trophoblasts in the presence of GSK126 than in its absence. We studied the same parameters in cells treated with the EZH2 siRNA (**Figures 3A–C** and **Supplementary Figure 3B**). In addition, we examined the proliferation of trophoblasts treated with EZH2 inhibitor or transfected with EZH2 siRNA in the presence of AD-MSC-derived exosome or Baf A1, and found that the trophoblast proliferation had the same change curve as the autophagy activity (**Figures 2D,E**, **3D,E**).

The EZH2 Overexpression in Trophoblasts Inhibited Autophagy and Activated mTOR Pathway Under Hypoxic Conditions

First, we confirmed that the overexpression plasmids of EZH2 could elevate the EZH2 mRNA levels in trophoblasts (**Figure 4A**). To determine whether the EZH2 plasmids present in trophoblasts were responsible for the observed effects, we examined the mTOR signaling and autophagy-related proteins in trophoblasts treated with EZH2 plasmids and AD-MSC-derived exosomes using Western blotting (**Figures 4B,C** and **Supplementary Figures 2A**, **3C**). The results showed that the EZH2 overexpression attenuated the AD-MSC-derived exosome-induced inhibition of mTOR signaling and decreased autophagy in trophoblasts. In addition, we examined the proliferation of trophoblasts treated with EZH2 overexpression plasmids in the presence of AD-MSC-derived exosome or Baf A1, and found that the trophoblast proliferation had the same change curve as the autophagy activity (**Figures 4D,E**). Thus, it revealed that EZH2 acted as a down-regulator of AD-MSC-derived exosome-induced autophagy and that mTOR signaling may act as an downstream autophagy inhibitor regulated by EZH2 in trophoblasts.

EZH2 Regulate Trophoblast Autophagy and Proliferation Through mTOR Signaling Pathway

To assess whether EZH2 mediated trophoblasts autophagy inhibition through the mTOR signaling pathway, the trophoblasts were transfected with EZH2-overexpression plasmids and treated with PQR620, a highly potent and selective mTOR inhibitor. Then, the expression levels of EZH2, mTOR and autophagy associated proteins in trophoblasts were evaluated by Western blotting. The Western blotting results confirmed that EZH2 overexpression in trophoblasts activated mTOR signaling and inhibited the autophagy in trophoblasts, and mTOR inhibitor attenuated the EZH2-mediated autophagy inhibition (**Figure 5A**). Moreover, the proliferation assays suggested that EZH2 overexpression and mTOR signaling activation inhibited the trophoblast autophagy and proliferation, and PQR620 treatment reversed such inhibition (**Figures 5B,C**). These results indicates that EZH2 in trophoblasts may regulate their autophagy through the mTOR signaling pathway.

AD-MSC-Derived Exosome-Mediated EZH2 Inhibition Increased Autophagy in Placental Explants

Next, the data from trophoblasts were compared with those from placental explant cultures, which are *in vivo* models of trophoblasts in which villous trophoblasts remain in a natural environment and under more physiologically relevant conditions than the traditional cell culture. The human placental explants were incubated in DMEM/F12 under hypoxic condition and then cultured in a medium containing AD-MSC-derived exosomes

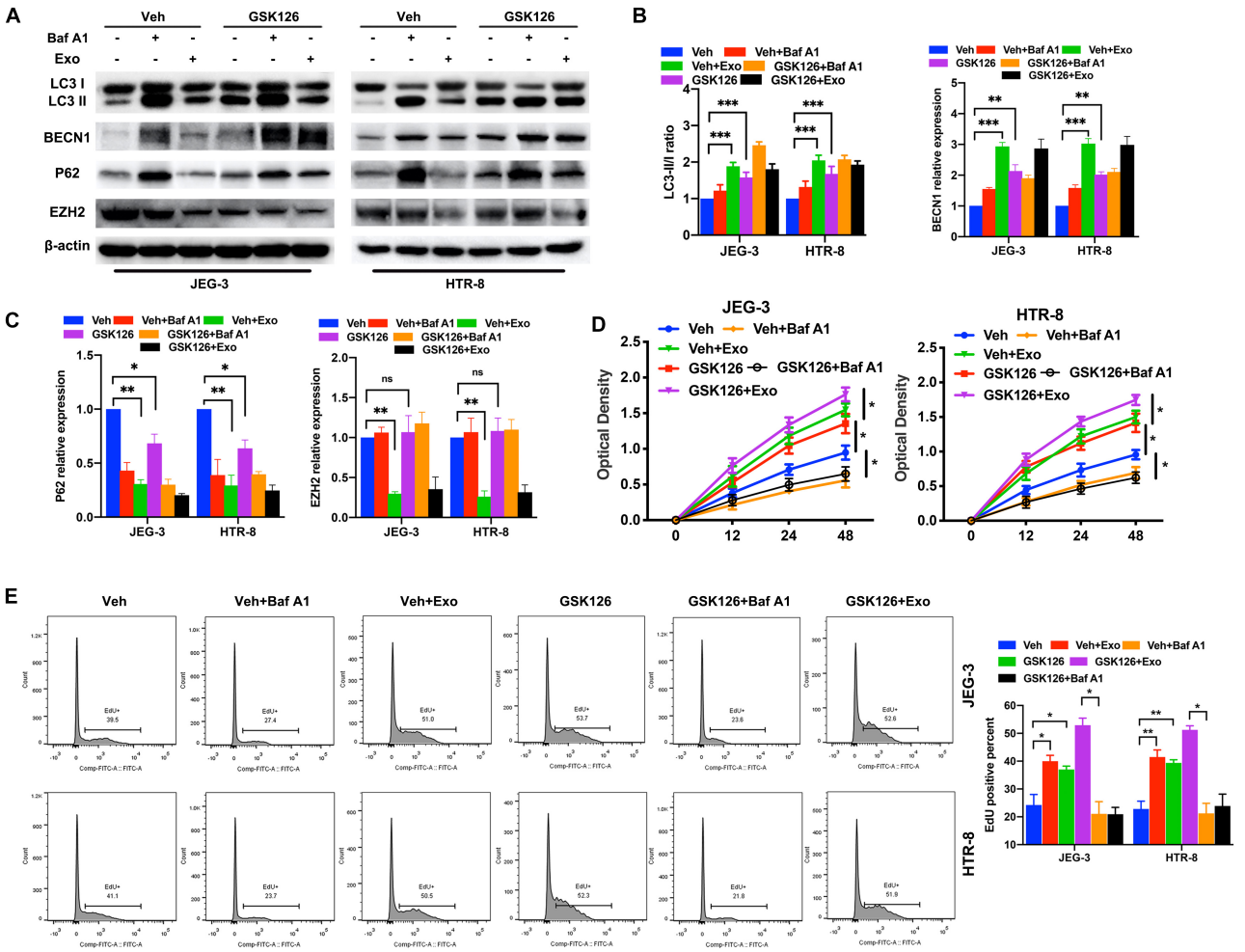

FIGURE 2 | The inhibition of EZH2 induced the increase of trophoblast autophagy and inhibition of mTOR pathway under hypoxic conditions. **(A–C)** The protein expression level of LC3, BECN1 and P62 levels were examined in JEG-3 and HTR-8 cells treated with GSK126, AD-MSC exosomes or Baf A1 for 24 h by western blot. The protein expression levels were quantified by densitometry. **(D)** Cell proliferation was evaluated by a CCK-8 assay. The trophoblast cell lines were treated with GSK126 or Baf A1 and with or without AD-MSC exosomes under hypoxic conditions. **(E)** The EdU positive percent of trophoblast cell lines were tested under hypoxic conditions by flow cytometry. $^{**}P < 0.01$, $^{*}P < 0.05$, $^{***}P < 0.001$.

transfected with or without EZH2 overexpression plasmids for 48 h. The Western blotting assays were used to determine the levels of EZH2 and autophagy associated proteins in the placental explants. While autophagy levels were significantly increased in the AD-MSC-derived exosomes-treated placental explants, and plasmids transfection attenuated the increase. Moreover, compared to the control explants, the EZH2 levels showed significant decrease in AD-MSC exosomes treated explants, and the plasmids transfection could elevate the EZH2 expression in explants (**Figure 6A**). Additionally, the immunohistochemical analysis showed a reduced EZH2 intensity in the villous cytotrophoblasts in AD-MSC-derived exosome-treated placental explants (**Figure 6B**).

Taken together, these data suggest that AD-MSC-derived exosomes inhibit the EZH2 expression and mTOR signaling pathway, and increase autophagic activity in cultured placental explants.

DISCUSSION

The amnion is the innermost avascular layer of the embryonic membranes and is an important source of promising cells that have therapeutic value. It has been used to improve a variety of medical conditions such as ophthalmology, skin injuries, and premature ovarian insufficiency. Its therapeutic effects are attributed to its inflammation-counteracting and immunomodulatory properties. Although the exact mechanisms are not clear yet, factors secreted by AD-MSCs are suggested to be the great contributors. In spite of the fact that the pathological mechanism of PE is illusive, dysfunction and hypoxia in trophoblasts were thought to be associated with preeclamptic placentas (Nakashima et al., 2017a,b). In this study, we provide evidences that AD-MSC-derived exosome-mediated inhibition of EZH2 expression and mTOR signaling pathway is the main mechanisms of autophagic activity regulation in human

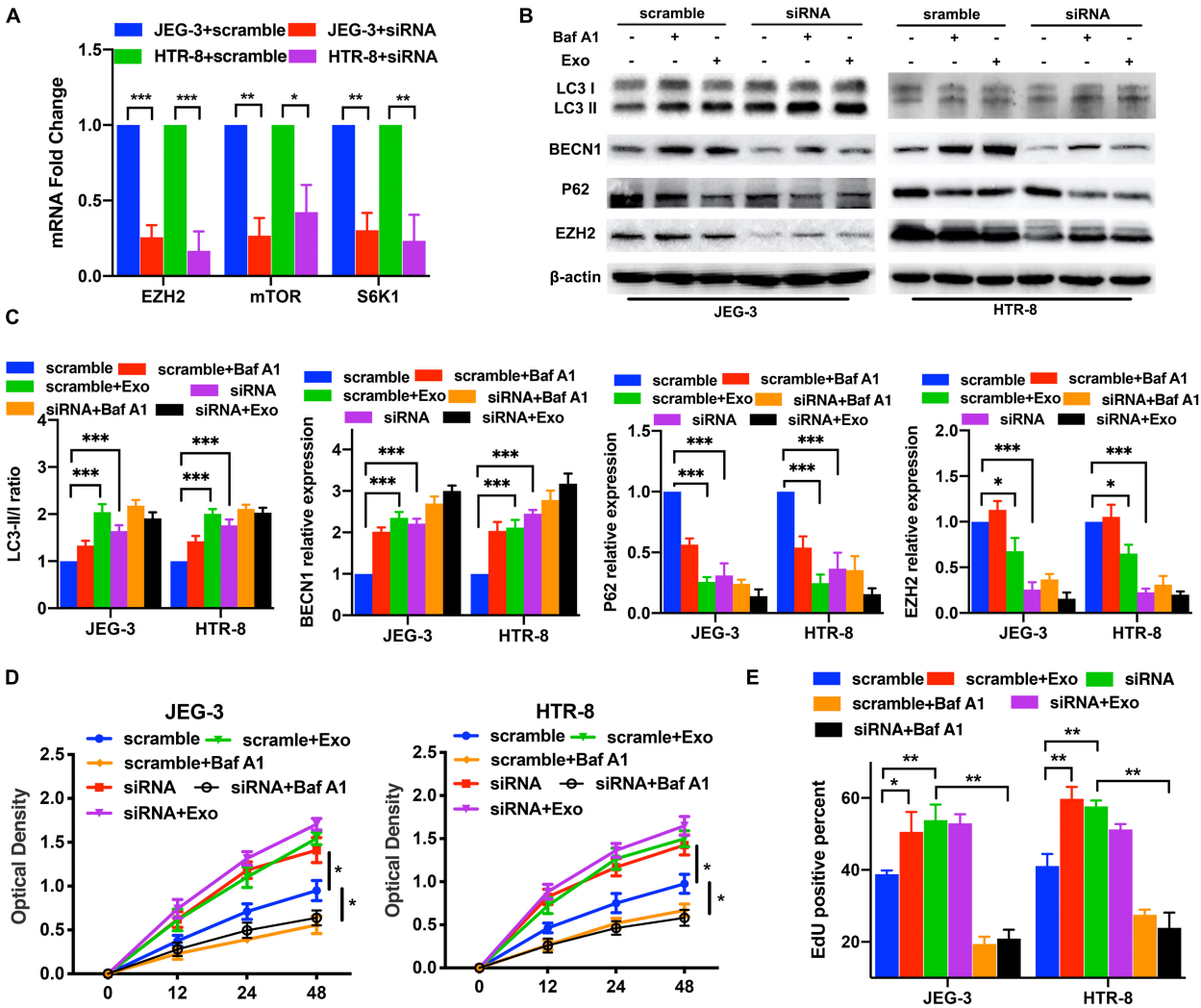

FIGURE 3 | The knockdown of EZH2 induced the increase of trophoblast autophagy and mTOR pathway inhibition under hypoxic conditions. **(A)** Significant decreases in EZH2, mTOR and S6K1 mRNA levels were tested in JEG-3 and HTR-8 cells transfected with EZH2 siRNA by qRT-PCR. **(B,C)** The trophoblast cell lines were transfected with a EZH2 siRNA under hypoxic conditions, and BECN1, P62 and LC3 expression levels were tested by western blotting analysis. Empty vector (scramble) cells served as controls. **(D,E)** Cell proliferation was evaluated by a CCK-8 assay and EdU flow cytometry. The two trophoblast cell lines were transfected with EZH2 siRNA with or without AD-MSC exosomes treatment under hypoxic conditions. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

villous trophoblasts and that it improves trophoblast survival in hypoxia conditions.

Autophagy was once deemed to occur in cytoplasm; however, recently, more and more evidences suggest that nuclear machineries (transcription factors, histone modification, microRNAs, etc.) are also involved in autophagy regulation (Boban and Foisner, 2016). Among them, epigenetic autophagy regulation has been given lots of attention. The epigenetic machinery can both modulate autophagy-related genes directly and affect some signal transduction genes that can in turn regulate autophagy, thereby influencing their transcription and autophagy subsequently (Shin et al., 2016). The methylation of DNA and modifications of histone were considered to be involved in autophagy regulation recently (Fullgrabe et al., 2014). The overexpression of EZH2 has been observed in a variety of cancers, and was correlated to cancer progression, metastasis and poor prognosis in various cancer types (Gan et al., 2018). In recent time, it is found that EZH2 might make a great contribution in autophagy (Shin et al., 2016). Particularly, downregulation of EZH2 was found as an epigenetic modulator of autophagy by regulating the mTOR pathway in the colorectal carcinoma (Wei et al., 2016). However, the mechanism involved in the epigenetic regulation of autophagy in trophoblasts is far from being fully known.

The mechanistic target of rapamycin (formerly mTOR, mammalian target of rapamycin) is a serine/threonine protein kinase that is evolutionarily conserved, playing a central role in regulating the cell growth, proliferation and survival, according to the condition of nutrition, signals of stress and growth factors. It is a crucial controller of fundamental biological

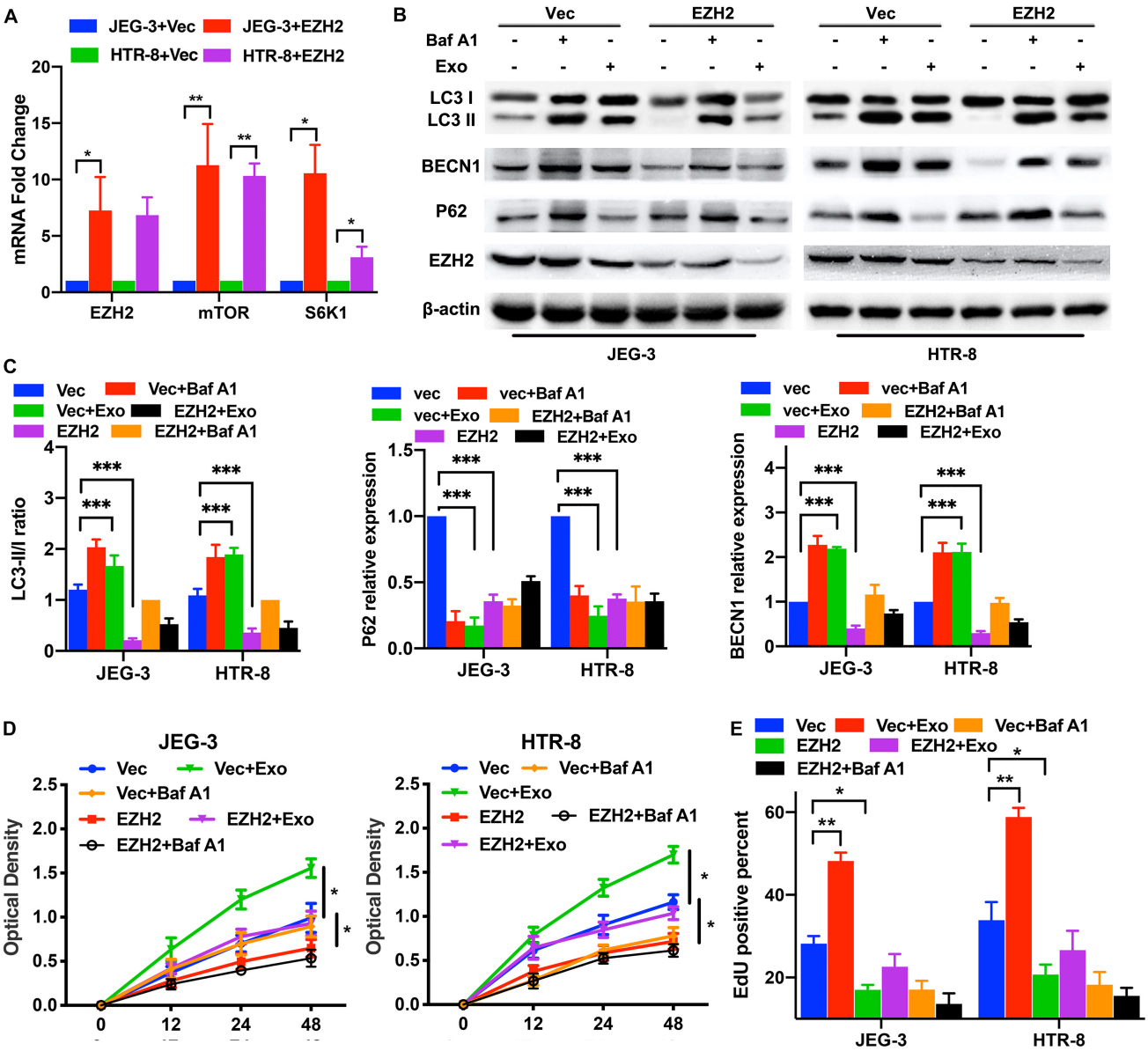

FIGURE 4 | EZH2 overexpression decreased trophoblast autophagy and proliferation through mTOR signaling pathway. **(A)** Significant increases in EZH2, mTOR and S6K1 mRNA were found in JEG-3 and HTR-8 cells transfected with EZH2 overexpression plasmid (EZH2) compared to empty vector (Vec)-transfected cells under hypoxic conditions by qRT-PCR. **(B,C)** The two trophoblast cell lines were transfected with EZH2 overexpression plasmid under hypoxic conditions, and BECN1, P62 and LC3 protein levels were tested by western blotting analysis. **(D,E)** Cell proliferation was evaluated by a CCK-8 assay and EdU flow cytometry. The trophoblast cell lines were transfected with a EZH2 overexpression plasmid or empty vector under hypoxic conditions. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

processes including lipid and glucose metabolism, autophagy, apoptosis, etc. Many researches have identified mTOR as a vital autophagy regulator, and deregulations of the mTOR pathway have been found to be involved in various pathological failures. On a molecular basis, autophagy regulates several signaling pathways that determines the death or continued survival of cells; nevertheless, the relationship between autophagy, EZH2 and mTOR pathways in trophoblasts is still undecided. The fate of trophoblasts, which were involved in hypertensive diseases during pregnancy, was affected by the autophagy regulation by AD-MSC-derived exosomes. In this study, the EZH2 expression was found to be reduced in trophoblasts treated with AD-MSC-derived exosomes, accompanied by the inactivation of the mTOR signaling pathway. These findings confirmed the curative effects of AD-MSC secretions and provided a novel insight into potential PE therapies.

In summary, it is revealed in our study that AD-MSCs promote the trophoblast proliferation and autophagy under hypoxic conditions *in vitro*, partially due to inactivation of mTOR signaling induced by the EZH2 downregulation. However, it

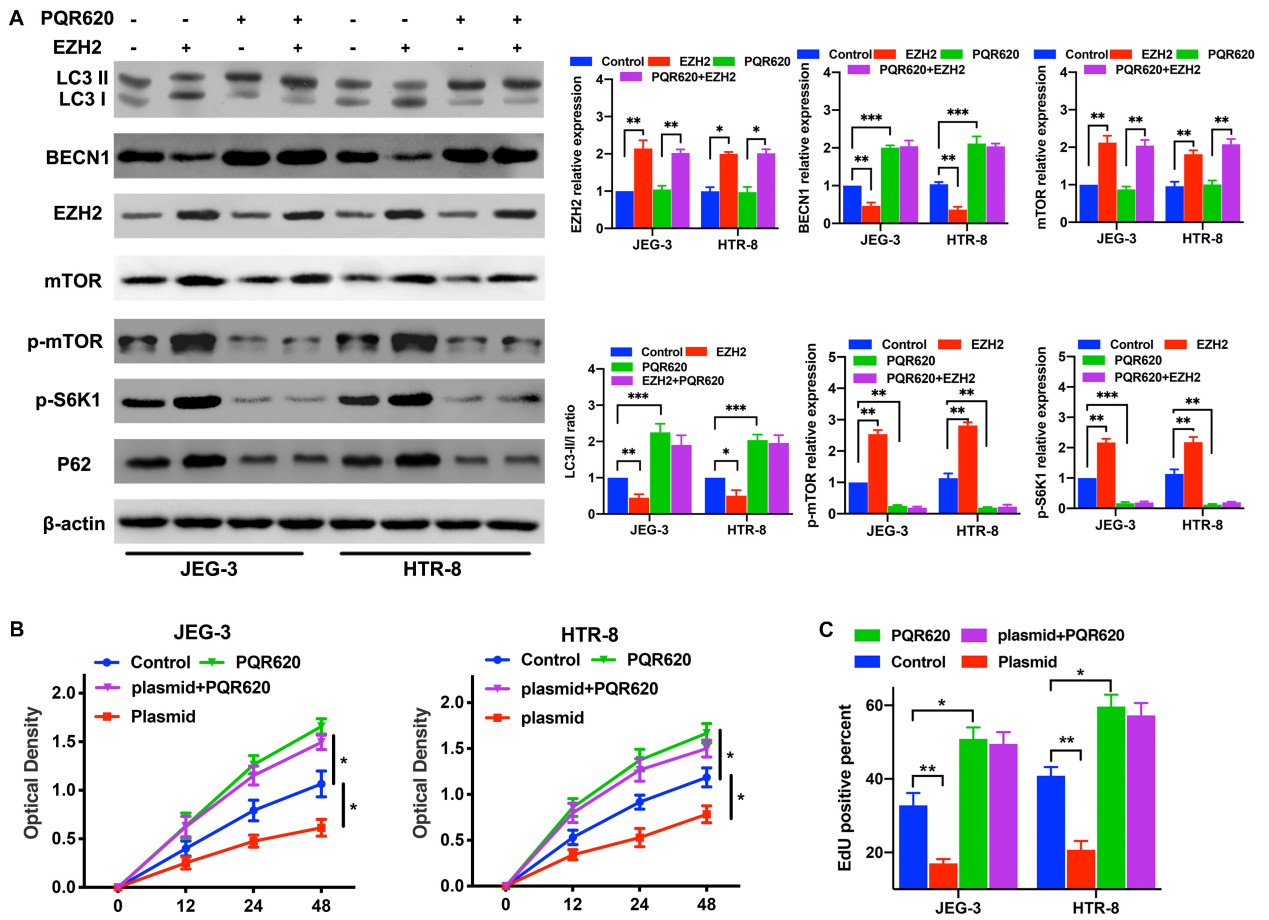

FIGURE 5 | EZH2 regulate trophoblast autophagy and proliferation through mTOR signaling pathway. **(A)** The levels of EZH2, p-mTOR, p-S6K1, and autophagy associated proteins were examined in JEG-3 and HTR-8 cells transfected with EZH2 overexpression plasmid and treated with m-TOR inhibitor PQR620 under hypoxic conditions by western blot. **(B,C)** Cell proliferation was evaluated by a CCK-8 assay and EdU flow cytometry. The trophoblast cell lines were treated with EZH2 overexpression plasmid and m-TOR inhibitor PQR620 under hypoxic conditions. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

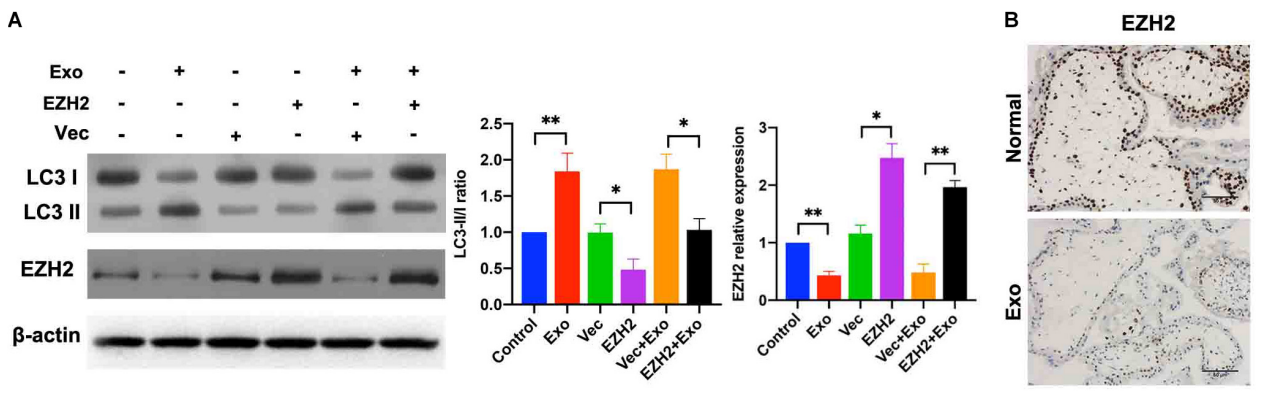

FIGURE 6 | AD-MSC-mediated EZH2 activation increased autophagy in placental explants under hypoxic conditions. **(A)** Placental explants were treated with AD-MSC exosomes transfected with EZH2 overexpression plasmids or Vec under hypoxic conditions, and EZH2, LC3 expression levels were tested by western blotting analysis. **(B)** Placental explants were treated with AD-MSC exosomes under hypoxic conditions, and EZH2 were tested by immunohistochemistry. Untreated placental explants served as controls (scale bar, 50 μm). *$P < 0.05$, **$P < 0.01$.

remains unclear how AD-MSCs influence other types of placenta-based cells *in vitro* or even *in vivo*; thus, further studies are needed on whether AD-MSCs affect them similarly. More studies on how EZH2 regulates autophagy will be important for a thorough understanding of the PE pathogenesis.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Affiliated Hospital of Qingdao University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

YC, YH, and YY conceived and designed the experiments. WC, YoL, YZ, JuZ, and HZ performed the experiments. WP, LX, JiZ, NZ, GG, and XW collected the samples. LL, JL, RH, CL, and KY analyzed the data. YC, HZ, and YaL wrote the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2020.545852/full#supplementary-material

Supplementary Figure 1 | Characterization of primary AD-MSCs derived from human placental tissues. **(A)** Representative photomicrographs of primary human AD-MSCs before confluence at passage 3. The cells were examined for osteogenic and adipogenic differentiation. Scale bar = 20 μm. **(B)** The purity of the isolated AD-MSCs and PE-AD-MSCs was examined by flow cytometry; AD-MSCs express CD44, CD73, CD90, and CD105, but lack CD34, CD45, CD146, IG1, and HLA-DR expression. **(C)** AD-MSC exosomes were visualized by electron microscopy (×30,000). Western blot examined the expression of exosomes marker TSG101 and CD63 in isolated AD-MSC exosomes. Typical imagines of internalizated exosomes derived from epidural AD-MSCs by trophoblasts JEG-3 at 4 h. Fluorescence microscopy images showing the internalization of exosomes by JEG-3 cells. Blue: Nucleus stained with DAPI. Red: PKH26-labeled exosomes. Scale bar: 50 μm. **(D)** The protein levels of H3K27me3, H3K4me3, H3 and EZH2 in JEG-3 cell treated with GSK126 concentration gradient for 6 h.

Supplementary Figure 2 | Effect of EZH2 siRNA, EZH2 inhibitor and overexpression plasmids on the EZH2 and mTOR pathways proteins expression of trophoblasts by western blot. **(A)** Protein expression of EZH2, mTOR, S6K1, p-mTOR, and p-S6K1 was examined in JEG-3 and HTR-8 cells treated with EZH2 siRNA, EZH2 inhibitor or overexpression plasmids by western blotting. **(B)** The levels of mTOR, S6K1, p-mTOR, and p-S6K1 in **Figure 1F** were quantified by densitometry. Data represent mean ± SD (error bars) of three independent experiments. $^{**}P < 0.01$, $^{***}P < 0.001$.

Supplementary Figure 3 | Levels of LC3-II in EZH2 siRNA, inhibitor and overexpression plasmids treated trophoblasts with or without ADSCs/Baf A1 by Immunofluorescence assays. **(A)** Expression of LC3-II in trophoblasts treated with GSK126, ADSCs exosomes and Baf A1 was examined by Immunofluorescence assays, and typical images were shown. **(B)** Expression of LC3-II in EZH2 siRNA transfected trophoblasts treated with or without ADSCs exosomes/Baf A1 was examined by Immunofluorescence assays, and typical images were shown. **(C)** Expression of LC3-II in EZH2 overexpression plasmids transfected trophoblasts treated with or without ADSCs exosomes/Baf A1 was examined by Immunofluorescence assays, and typical images were shown (scale bar, 25 μm).

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frontiers in Cell and Developmental Biology

REVIEW
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Molecular Pathways Modulated by Mesenchymal Stromal Cells and Their Extracellular Vesicles in Experimental Models of Liver Fibrosis

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End-stage liver fibrosis is common to all chronic liver diseases. Since liver transplantation has several limitations, including lack of donors, immunological rejection, and high medical costs, therapeutic alternatives are needed. The administration of mesenchymal stromal cells (MSCs) has been proven effective in tissue regeneration after damage. However, the risk of uncontrolled side effects, such as cellular rejection and tumorigenesis, should be taken into consideration. A safer alternative to MSC transplantation is represented by the MSC secretome, which retains the same beneficial effect of the cell of origin, without showing any considerable side effect. The paracrine effect of MSCs is mainly carried out by secreted particles in the nanometer range, known as extracellular vesicles (EVs) that play a fundamental role in intercellular communication. In this review, we discuss the current literature on MSCs and MSC-EVs, focusing on their potential therapeutic action in liver fibrosis and on their molecular content (proteins and RNA), which contributes in reverting fibrosis and prompting tissue regeneration.

Keywords: mesenchymal stem cell, collagen, α-SMA, hepatic stellate cell, fibrosis, inflammation, microvesicles, exosomes

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Abbreviations: CLD, chronic liver disease; ECM, extracellular matrix; DAMPs, damage-associated molecular patterns; HSC, hepatic stellate cell; α-SMA, alpha-smooth muscle actin; EMT, epithelial-to-mesenchymal transition; PDGF, platelet-derived growth factor; TGF-β, transforming growth factor beta; TNF-α, tumor necrosis factor alpha; IL, interleukin; CCL2, chemokine (C–C motif) ligand 2; STAT, signal transducer and activator of transcription; PI3K, phosphatidylinositol 3-kinase; MMP, metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; SMAD, small mother against decapentaplegic; NASH, non-alcoholic steatohepatitis; MSC, mesenchymal stromal cell; EV, extracellular vesicle; HpSCs, hepatic stem/progenitor cells; CCl_4, carbon tetrachloride; BM-MSC, bone marrow-derived MSC; IFN-γ, interferon gamma; TAA, thioacetamide; PGE2, prostaglandin E2; IDO, indoleamine 2,3-dioxygenase; NO, nitric oxide; HFD, high fat diet; MCDD, methionine- and choline-deficient diet; α-FP, alpha-fetoprotein; CK-18, cytokeratin-18; GFAP, glial fibrillary acidic protein; APAP, acetaminophen; hMSC-HC, human MSC differentiated into hepatocyte-like cell *in vitro*; hDPSC, human deciduous pulp stem cell; VEGF, vascular endothelial growth factor; HGF, hepatocyte growth factor; UC-MSC, umbilical cord-derived MSC; MFGE8, milk factor globule EGF; AMSC, amniotic fluid-derived MSC; MVBs, multivesicular bodies; ESCRT, endosomal sorting complex required for transport; ALIX, apoptosis-linked gene-2 interacting protein X; ARRDC1, arrestin domain-containing protein-1; TSG101, tumor susceptibility gene 101; ROCK, Rho-associated protein kinase; ARF6, ADP-ribosylation factor 6; UC-PVC, umbilical cord-derived perivascular cell; IGF-1, insulin growth factor 1; iNOS, inducible nitric oxide synthase; CP-MSC, chorionic plate-derived MSC; ESC, embryonic stem cell; ADSCs, adipose tissue-derived MSC; CCNG1, cyclin G1; IGF1R, insulin-like growth factor receptor 1; P4HA1, prolyl-4-hydroxylase a1; PPAR, peroxisome proliferator-activated receptor; HLSC, human liver stem cell; Ltbp1, latent-transforming growth factor beta-binding protein 1.

INTRODUCTION

Chronic liver disease (CLD) may be caused by different types of injury (viral infection, alcohol abuse, NASH, ischemic injury, chemical compounds, autoimmune and genetic diseases, etc.), and it is one of the major global health problems that causes about 2 million deaths per year worldwide (Asrani et al., 2019; Roehlen et al., 2020). Fibrosis is the main characteristic of CLD and is due to an excessive ECM accumulation, which compromises the normal morphology and function of the liver. Excessive ECM deposition is due to a persistent activation of myofibroblasts that proliferate and produce different matrix components (type I and III collagen, fibronectin, laminin, and proteoglycans) (Roehlen et al., 2020).

Hepatocytes are proliferative cells that support the physiological renewal of liver parenchyma. In case of injury, proliferation is due to the activation of resident HpSCs, which are quiescent during physiological turnover of the organ (Ibrahim et al., 2018). Different studies have observed only a marginal contribution of these resident progenitor cells in ameliorating the damage in several models of hepatocellular injury. In particular, the HpSCs are not able to totally regenerate the hepatocytes damaged in CLD, especially in the presence of a massive destruction of the liver tissue (Español-Suñer et al., 2012). For this reason, an exogenous treatment that supports the regeneration of the altered tissue is demanded.

A possible therapeutic option is the use of MSCs that can be isolated from various adult tissues, such as bone marrow (BM), adipose tissue, muscle, periosteum, umbilical cord, blood, and liver (Puglisi et al., 2011). MSCs were originally described as a rare population of cells in the BM with fibroblast-like morphology (Friedenstein et al., 1974) and a characteristic pattern of cell-surface antigens (Pittenger et al., 1999). Besides their self-replicating ability, MSCs can differentiate in mesenchymal (osteoblasts, chondrocytes, adipocytes, and myocytes) and non-mesenchymal cells (cardiac, neural, renal, and hepatic cells) (Krause, 2002; Alhadlaq and Mao, 2004). These peculiar properties combined with the immunoregulatory activity of MSCs make these cells ideal candidates for regenerative therapy of various diseases, including liver fibrosis (Eom et al., 2015; Han et al., 2019).

In this review, we summarize recent findings in the field of MSCs application as anti-fibrotic strategy and on molecular pathways modulated by MSCs and their secretome, in particular by EVs.

CELLS AND MOLECULAR PATHWAYS INVOLVED IN HEPATIC FIBROSIS

Different types of hepatic cells contribute to fibrosis development. Hepatocyte death is the initial event in liver diseases that conducts to damage progression. Dead hepatocytes release different molecules (intracellular proteins, ATP, nucleic acids, mitochondrial or nucleic compounds) collectively named DAMPs (Mihm, 2018). These intracellular compounds negatively influence the neighboring cells, such as HSCs and Kupffer cells, favoring cell activation and fibrosis development. Moreover, a pro-fibrogenic signal can be triggered by apoptosis of hepatocytes by the activation of Fas death receptor (Feldstein et al., 2003) and by the release of apoptotic bodies. These can be absorbed by Kupffer cells and by HSCs to activate pro-fibrogenic signals (Canbay et al., 2003; Zhan et al., 2006).

Hepatic stellate cells are the key players in hepatic fibrosis development, since they represent the major source of myofibroblast precursors. In physiological conditions, HSCs are perisinusoidal non-proliferating cells characterized by numerous lipid droplets containing vitamin A in the cytoplasm. Different types of injury may induce HSC activation. In this case, HSCs start to proliferate, acquire a contractile myofibroblast phenotype, express α-SMA, and produce ECM components and pro-inflammatory cytokines (Mederacke et al., 2013). Other important sources of myofibroblasts may be portal fibroblasts (Wells et al., 2004), BM-derived cells (Forbes et al., 2004), and cells derived from hepatocytes or cholangiocytes by EMT (Zeisberg et al., 2007). In addition, the production of pro-inflammatory cytokines induces the recruitment and the activation of other important cells in fibrosis development, such as platelets, endothelial cells, and infiltrating immune cells that may amplify the pro-fibrogenic environment and contribute to support HSC and myofibroblast activation state. Hepatic macrophages, which can be liver resident (Kupffer cells) or monocyte-derived (Krenkel and Tacke, 2017), play a central role in this fibrosis development. Macrophages may be classified into pro-inflammatory macrophages (M1) and immunoregulatory macrophages (M2). Hepatic macrophages may shift from M1 to M2 phenotypes in response to different stimuli, and different macrophage subpopulations may coexist and contribute to different phases of fibrosis (Sun et al., 2017).

A complex network of cytokines and pathways is responsible for HSC activation and for induction of fibrogenic alterations. The most important growth factors and pathways involved in fibrogenesis are the PDGF, the TGF-β, the inflammasome NLRP3–caspase-1, and the Wnt/β-catenin signaling pathway (Dewidar et al., 2019; Roehlen et al., 2020). Moreover, resident immune cells produce under stimulation the TNF-α, the interleukins (IL-6 and IL-1α), and the CCL2, that trigger the activation of Kupffer cells and the recruitment of monocyte-derived macrophages from blood (Roehlen et al., 2020). In physiological conditions, PDGF is produced by platelets. Different types of liver injury induce the production of PDGF also by Kupffer cells, endothelial cells, and activated HSCs, and PDGF receptor is expressed by HSCs (Campbell et al., 2005; Hayes et al., 2014). The binding of PDGF to its receptor triggers the activation of several signaling pathways, including the PI3K/Akt, the JAK/STAT, and the Ras/Raf system. These different pathways regulate downstream the expression levels of the key pro-fibrotic genes, such as type I collagen α1 chain, MMPs, tissue inhibitors of metalloproteinases (TIMPs), and also apoptosis regulators (Bcl-2) that favor the survival and proliferation of myofibroblasts (Ying et al., 2017). Endothelial cells, macrophages, and hepatocytes can synthesize TGF-β, as a latent precursor. The inactive form of TGF-β, bound to the latency-associated protein, is stored in the ECM. TGF-β becomes active when cleaved by a

specific protease. The active form binds to TGF-β receptor type II, which recruits the TGF-β receptor type I, with downstream activation of SMAD proteins. In particular, the activation of SMAD3 by phosphorylation at its C-terminus is considered the main fibrogenic pathway (Liu et al., 2006; Fabregat et al., 2016). Furthermore, the activation of the SMAD3-dependent TGF-β signaling pathway in hepatocytes contributes to fibrosis development, especially in NASH, by inducing hepatocyte death and lipid accumulation. SMAD6 and SMAD7, which negatively regulate TGF-β signaling, are considered as anti-fibrotic factors (Yang et al., 2014).

Another important pathway involved in hepatic fibrosis development is the Wnt/β-catenin signaling. β-Catenin is an adhesion molecule that can also act as a transcription factor. After activation, Wnt binds the receptor Frizzled and forms a complex, which inhibits β-catenin degradation causing its translocation to the nucleus that activates target genes transcription by recruiting cyclic AMP-response element binding (CREB) protein (Nishikawa et al., 2018). In hepatic damage, the Wnt signaling pathway is activated in the HSCs and may contribute to fibrosis development by upregulating α-SMA and collagen expression (Berg et al., 2010).

Despite the increasing number of studies deeply investigating the molecular mechanisms of liver fibrosis development, an approved drug to counteract liver fibrosis is still missing. Anti-fibrotic therapies have to focus on different mechanisms of action: hepatocyte protection, the inhibition of HSC activation, the consequent deposition of fibrotic molecules, and the modulation of inflammation.

MSC TREATMENT COUNTERACTS LIVER FIBROSIS DEVELOPMENT

Several studies demonstrated that the injection of MSCs improved liver fibrosis and enhanced liver functionality by reducing hepatocyte apoptosis, prompting hepatocyte regeneration, and regulating inflammatory responses in different animal models of CLD (**Table 1**). In particular, numerous published results demonstrated the reduction of TGF-β1 and α-SMA gene expression in liver tissue after treatment with MSCs (Rabani et al., 2010; Jang et al., 2014; Winkler et al., 2014; Idriss et al., 2018; Fathy et al., 2020; Sun et al., 2020).

In the CCl_4-induced CLD model, the administration of murine BM-derived-MSCs reduced fibrosis, ameliorated the hypoxic liver microenvironment, and improved liver function. These beneficial effects were correlated with a modulation of the TGF-β1/SMADs signaling pathway in liver cells. In particular, BM-MSCs reduced TGF-β1 and SMAD3 expression and increased SMAD7 expression (Zhang et al., 2015, 2019). SMAD7 can be regulated by different stimuli, including TGF-β, IFN-γ, and TNF-α. The downregulation of SMAD7 expression is associated with both tissue fibrosis and inflammatory disease; instead, its over-expression antagonizes TGF-β-mediated fibrosis and inflammation (Yan et al., 2009).

Another aspect that characterizes in a distinctive manner liver fibrosis is the induction of collagen deposition, with a consequent complete remodeling of the ECM. The interstitial ECM is mainly composed of structural fibrils of type I and type III collagen and in a minor quantity of type V collagen (Karsdal et al., 2020). The analysis of gene expression profile of ECM in MSC-treated fibrotic animals indicated the reduction of the deposition of collagen in the area of injury. The pro-collagen gene expression significantly decreased when MSCs were administrated in liver fibrosis models generated by the injection of CCl_4 and of TAA chemical drugs (Jang et al., 2014; Farouk et al., 2018), especially when the transplantation of cells occurred *via* the portal vein rather than by the tail vein (Truong et al., 2016). Besides, IL-6 stimulates the activation of STAT3 and increases collagen mRNA expression in HSCs; the phosphorylation of STAT3 activates the TGF-β cascade through SMAD3 activation (O'Reilly et al., 2014). In this framework, Zhang et al. (2019) demonstrated that BM-MSCs reduced fibrosis by modulating the TGF-β1/SMADs signaling. Yuan et al. (2019) showed that the low amount of collagen deposition was related to low IL-6 mRNA levels and the reduction was evident especially for type III collagen α1. Several studies reported the involvement of IL-17A in liver fibrosis (Zepeda-Morales et al., 2016), since the IL-17 receptor complex IL-17RA/IL-17RC induced in HSC the activation of STAT3, which led to increased collagen mRNA expression. After MSC transplantation, the expression of fibrogenic type I collagen α1 mRNA decreased in liver tissue. Moreover, a gradual reduction of the mRNA expression of IL-17a, IL-17f, and IL-17ra and IL-17rc receptors was observed in the BM-MSC-treated group (Farouk et al., 2018).

The reduction in pro-collagen gene expression often correlates with increased secretion of collagen-degrading MMPs (Rengasamy et al., 2017; Du et al., 2018). In fact, the administration of MSCs induces the reduction of fibrosis by increasing the expression of MMP-9, which degrades the ECM (Tanimoto et al., 2013) and modulates genes involved in matrix remodeling, such as MMP-2 and TIMP-1 (Zhao et al., 2012; Rengasamy et al., 2017; Du et al., 2018; Khalifa et al., 2019; Iwanaka et al., 2020).

Mesenchymal stromal cells can influence the fibrosis development indirectly through the reduction of the hepatic inflammatory state. In fact, MSCs display an immunoregulatory activity by preventing the maturation of immune cells. In particular, they inhibit the proliferation of dendritic, T helper-1, and natural killer cells and induce the activation of M2 macrophages through the production of PGE2, IDO, and NO and secretion of anti-inflammatory ILs, such as IL-10 (Aggarwal and Pittenger, 2005). Anti-inflammatory effects of MSCs in liver tissue were evaluated by measuring the expression levels of pro-inflammatory cytokines in different animal models of CLD, such as HFD, MCDD, CCl_4 infusion, and TAA administration (Zhao et al., 2012; Nasir et al., 2013; Idriss et al., 2018; Bruno et al., 2019). These studies demonstrated that TNF-α, IL-6, and other pro-inflammatory cytokines [IL-1β, TGF-β1, INF-γ, and monocyte chemoattractant protein-1 (MCP-1)] were downregulated in the liver of treated mice (Zhao et al., 2012; Nasir et al., 2013; Idriss et al., 2018; Bruno et al., 2019; Choi et al., 2019; Kojima et al., 2019).

TABLE 1 | Anti-fibrotic effect of MSCs from different sources in chronic liver injury.

Type of cells	Source	Liver fibrosis model	Mechanism of action	References
Murine MSCs	BM mononuclear cell fraction selected by plastic adherence	Intraperitoneal (i.p.) administration of a 1.0 ml/kg dose of CCl4 twice a week for 4 weeks	Reduction of collagen deposition by downregulating α-SMA and TIMP-1 gene expression	Rabani et al., 2010
Murine hepatocyte-differentiated BM-MSCs (in the presence of HGF, FGF4, and EGF)	Tibia and femur of Sprague–Dawley (SD) rats	0.5 ml/kg CCl4 was injected subcutaneously into adult male SD rats (320 ± 20 g) twice a week for 4 weeks	Improvement of liver function by modulating the gene of ECM remodeling (MMP-2, MMP-9, and TIMP-1), reducing the expression of IL-1β, IL-6, TNFα, and TGF-β, and increasing IL-10 and HGF	Zhao et al., 2012
Human BM-MSCs and human MSCs	Purchased from Lonza	CCl4 dissolved in corn oil (1:3) twice a week for 6 weeks for the last 4 weeks	Fibrosis reduction by improvement of MMP-9, which degrades the ECM, and downregulation of αSMA, TNFα, and TGF-β, markers of activated HSCs	Tanimoto et al., 2013
Murine MSCs and hepatocytes	Tibia and femur of 2-month-old C57BL/6 mice	*In vitro*: hepatocytes were seeded on a 6-well collagen-coated plate (1 × 104 cells/cm^2) and were subjected to injury with 3 mM and 5 mM CCl4. *In vivo*: female C57BL/6 mice (6–8 weeks old) intraperitoneally injected with 1 ml/kg CCl4 in olive oil (1:1) for 4 weeks.	*In vitro*: reduction of apoptotic markers, such as Bax, caspase-3, NF-κB, IL-6, and TNF-α, and increased levels of anti-apoptotic marker Bcl-xl. *In vivo*: increased expression of Bcl-xl and reduction of expression levels of apoptotic markers Bax, caspase-3, NF-κB, and TNF-α	Nasir et al., 2013
Human BM-MSCs	Posterior iliac crest of healthy donors	i.p. injections of TAA (300 mg/kg body weight) twice a week for 12 weeks in SD rats	Recovery from TAA induced fibrosis by decreasing TGF-β1, type I collagen, and α-SMA expression and modulating the TGF-β1/SMAD signaling pathway	Jang et al., 2014
Human hepatocyte-differentiated BM-MSCs	Knee or hip joint of human donors	Immunodeficient male Pfp/Rag2–/– mice that underwent 1/3 partial hepatectomy after 42 days of MCDD	Presence of human hepatocyte-like cells in the mouse liver parenchyma attenuating inflammation markers (TNFα). Reduction of the expression of α-SMA and type I collagen mRNA	Winkler et al., 2014
Human BM-MSCs	BM of human healthy donors	*In vitro* model: co-culture of BM-MSCs with HSCs in transwell condition for 24, 48, and 72 h	Inhibitory effect of BM-MSCs on HSC proliferation and induction of the apoptosis through the inhibition of the TGF-β1/SMAD pathway in HSCs	Zhang et al., 2015
Murine BM-MSCs	Femur of healthy Swiss mice	Male Swiss mice treated by 1.0 ml/kg CCl4 via oral administration 3 times/week (every 2 days) for 11 weeks	Inhibition of fibrogenesis with the reduction of integrin, TGF-β1, and pro-collagen expression	Truong et al., 2016
Adult human BM-MSCs and neonatal human Wharton's jelly (WJ)-MSCs	BM mononuclear cells from three independent donor aspirations (BM-MSCs) and fresh umbilical cords collected from full-term births (WJ-MSCs)	SD rats i.p. injected with CCl4 at a dose of 2 ml/kg (CCl4:olive oil = 1:1) twice a week for the first 2 weeks, followed by 1 ml/twice a week for the next 6 weeks	Reduction of liver collagen content and improvement of liver architecture, by secreting fibrinolytic metalloproteases, such as MMP-1 and MMP-2	Rengasamy et al., 2017
Rat BM-MSCs	Tibia and fibula of white albino rats	0.2 ml/100 g of CCl4 liquefied in castor oil (40 ml/L) subcutaneously injected twice weekly for 6 weeks in male 6-week-old white albino rats	Recovery of liver function and improved liver fibrosis with prolonged presence of transplanted BM-MSCs in the liver: reduction in the expression of pro-inflammatory cytokines (IL-1β, IL-6, and IFN-γ) and of pro-fibrogenic factors (TGF-β1, α-SMA, and CTGF) and increase in the expression of anti-fibrogenic factors (CK-18 and HGF)	Idriss et al., 2018
Rat BM-MSCs transfected with human MMP-1	BM of SD rats	CCl4 administration in rats at a dose of 1 ml/kg twice/week for 8 weeks	Degradation of hepatic collagen due to significant increased MMP-1 level and suppression of TIMP-1	Du et al., 2018
Rat BM-MSCs	Tibia and femur of rats	Rats i.p. injected with CCl4 (1 ml/kg) dissolved in paraffin oil, twice a week, for 6 weeks (12 doses)	Significant downregulation of Col1a1, AFP, and STAT3 and STAT5 gene, whereas significant improvement of Alb expression	Farouk et al., 2018
Human BM-MSCs cultured under hypoxic (5% O_2; hypoMSCs) and normoxic (21% O_2; norMSCs) conditions	Normo: Poietics human MSCs (passage 2) purchased from Lonza. Hypo: StemPro BM-MSCs from Thermo Fisher Scientific	*In vitro*: induced BM-derived co-cultured with MSCs in Transwell 6-well plates for 72 h. *In vivo*: 8-week-old C57BL/6 male mice injected with CCl4 i.p. twice weekly over a 12-week period	Induction of anti-inflammatory markers CD206 and Ym-1 in hypoMSC-treated macrophages. Downregulation of the pro-inflammatory markers TNFα and MCP-1	Kojima et al., 2019

(Continued)

TABLE 1 | Continued

Type of cells	Source	Liver fibrosis model	Mechanism of action	References
BM-MSCs labeled with super-paramagnetic iron oxide nanoparticles	Femur of male SD albino rats	SD albino rats i.p. injected with CCl4 for 8 weeks	ECM degradation by increased MMP-1 and decreased TIMP-1	Khalifa et al., 2019
Murine BM-MSCs	Tibia and femur of mice C57BL/6	C57BL/6 mice injected with CCl4 (40% in olive oil) at a dose of 1 ml/kg twice/week for 12 weeks	Amelioration of the hypoxic liver microenvironment, improvement of the liver function, and reduction of fibrosis by modulating the TGF-β1/SMADs signaling pathway: reduction of TGF-β1 and SMAD3 expression and increased SMAD7 expression	Zhang et al., 2019
Human ADSCs	Human adipose tissue-derived stromal vascular fraction	Male NOD/SCID mice (NOD.CB17-Prkdcscid/J strain) i.p. injected with 200 mg/kg with TAA 2 times/week for 4 weeks	Induction of liver regeneration and amelioration of fibrosis and inflammation with downregulation of IL-1α, IL-6, and TNF-α and increased expression of HGF and VEGF-A	Choi et al., 2019
Rat BM-MSCs + VEGF	Tibia and femur of 2-week-old SD rats	8-week-old rats i.p. injected with 40% CCl4 at 0.3 ml/100, twice per week, for 12 weeks. VEGF group and the BMSC + VEGF group i.v. injected with VEGF over-expressing adenovirus at 3 × 109 ifu (0.5 ml), once a week for 4 weeks	Low amount of collagen deposition related to low IL-6 mRNA levels; high levels of VEGF and VCAM-1 expression in the hepatic sinusoidal endothelial cells	Yuan et al., 2019
HLSCs	Liver fragment processed in Good Manufacturing Practice (GMP) procedure	NASH induced by MCDD	Significant improvement of liver function and morphology, at histological and molecular levels, by persistence of undifferentiated HLSCs in the liver that induce reduction of α-SMA, type I collagen, and TGF-β expression	Bruno et al., 2019
hDPSCs	Deciduous teeth of healthy pediatric donors	C57BL/6J male, 8-week-old mice i.p. injected with 0.5 mg/kg of CCl4 in olive oil twice a week for 4 weeks	Liver regeneration induced by the *in situ* transformation of the transplanted hDPSCs, with reduced expression of ACTA2, Col1a1, and liver fibrosis-related genes and proteins: MMP-2, MMP-3, TIMP-1, TIMP-2, and TGF-β	Iwanaka et al., 2020
BM-MSCs with recombinant adeno-associated virus expression vector encoding human HGF genome sequence (rAAV-HGF)	Stem Cell Bank of the Chinese Academy of Sciences (CAS)	SD rats fed with 5% ethanol and subcutaneously injected with 40% CCl4 diluted 1:1 (v/v) in olive oil (0.5 ml/kg) 3 times/week for 9 weeks	Reduction of fibrotic structure related to low expression of α-SMA, collagen I, and vimentin transcripts	Sun et al., 2020
Rat ADSCs incubated with eugenol in olive oil (10 mg/ml)	Adipose tissue of 2-month-old male rats	SD-1 rats i.p. injected with 1 ml/kg of CCl4 diluted in olive oil 1:1 (v/v) twice a week for 6 weeks	Amelioration of liver function, reduction of fibrotic markers (type III collagen, hyaluronic acid, hydroxyproline) and inflammatory cytokines (TNF-α, IL-1β, and IL-6), by decreasing the mRNA levels of type 1 collagen, α-SMA, and TGF-β genes	Fathy et al., 2020

Moreover, treatment with MSCs increased the production of the anti-inflammatory IL-10 that may modulate the expression of α-SMA, collagen I, and TGF-β in target cells both *in vitro* and *in vivo* (Rabani et al., 2010; Idriss et al., 2018; Choi et al., 2019).

Although MSC transplantation has shown beneficial effects in liver fibrosis, several issues must be carefully considered, including the injected cell dose and the timing of treatment. The anti-fibrotic effect of MSCs seems to be dose-dependent: in fact, compared with lower doses, higher cell doses showed a significant reduction in collagen release (Hong et al., 2014). Furthermore, the anti-fibrotic effect of MSCs in the liver is more evident when MSCs are administered in the earlier stages of injury (Zhao et al., 2005), whereas no beneficial effects are observed when MSCs are injected after long-term injury (Popp et al., 2007; Carvalho et al., 2008).

MSC ENGRAFTMENT AND DIFFERENTIATION INTO LIVER TISSUE

A few studies support the idea of the engraftment and differentiation of MSCs in the damaged tissue (di Bonzo et al., 2008; Stock et al., 2014; Iwanaka et al., 2020). In a model of intoxication of the liver caused by a sub-lethal dose of acetaminophen (APAP) and treated with human MSCs differentiated into hepatocyte-like cells *in vitro* (hMSC-HCs), the engrafted cells were detected in liver section after long-term transplantation (7 weeks after treatment). In particular, hMSC-HCs were localized in the periportal areas of the liver damaged by APAP, expressing both human albumin and HepPar1, a mitochondrial antigen of hepatocytes, indicating an *in situ* differentiation of hMSC-HCs into hepatic precursors. The engraftment of hMSC-HCs did not cause an increment in the

collagen content with respect to untreated APAP animals (Stock et al., 2014). Recently, also in CCl_4-injured liver, it was reported that the hepatic regeneration is due to *in situ* differentiation of the transplanted hDPSCs. The regenerative effect was correlated with the reduced gene expression of α-SMA, collagen 1, and other fibrosis-related genes and proteins, such as MMP-2, MMP-3, TIMP-1, TIMP-2, and TGF-β (Iwanaka et al., 2020).

In contrast, some authors have reported that, after *in vivo* injection, BM-MSCs can differentiate into myofibroblasts, thus contributing to the progression of liver fibrosis (di Bonzo et al., 2008; Baertschiger et al., 2009). di Bonzo et al. (2008) demonstrated that human BM-MSCs were able not only to engraft around the portal tract of both normal and CCl_4-injured liver of NOD/SCID mice but also to differentiate into hepatic cells in the *in vivo* acute liver injury model (di Bonzo et al., 2008). In the chronic liver injury model, the percentage of cells expressing the human leukocyte antigen (HLA)-I was significantly higher than in the acute setting; however, a significant number of human cells co-expressed markers of myofibroblast-like cells (α-SMA or glial fibrillary acidic protein) and were located around fibrotic areas, indicating a pro-fibrotic effect of MSCs. These evidences correlated in part with the results obtained by Baertschiger et al. (2009) in a murine model of partial hepatectomy associated with the inhibition of endogenous liver regeneration by retrorsine treatment. Following intrasplenic injection of BM-MSCs, the engraftment of these cells was not achieved in the liver. However, after intrahepatic injection, BM-MSCs permanently engrafted into the liver and might contribute to fibrosis by differentiating into myofibroblasts (Baertschiger et al., 2009). Besides, by comparing the *in vivo* effect of adult BM-MSCs with the effect of pediatric BM-MSCs, no difference in MSC engraftment and no evidence of MSC differentiation into hepatocytes were observed (Baertschiger et al., 2009). Whether the transplanted MSCs have a positive or negative effect on resident liver cell populations (HSCs, immune cells, hepatocytes) is an important aspect that needs to be considered (Kallis and Forbes, 2009). Further studies are required to better understand those mechanisms that could induce MSCs or resident liver cells to produce scar or ECM-degrading substances.

ANTI-FIBROTIC AND ANTI-INFLAMMATORY EFFECTS OF MSC SECRETOME

In addition to direct differentiation of MSCs into liver cells, MSCs exert pro-regenerative and anti-fibrotic effects in liver tissue by inducing the proliferation of resident mature hepatocytes or of progenitor cells through the secretion of paracrine factors. Analysis of gene expression of different sources of MSC revealed that MSCs produce several molecules that can inhibit the activation of HSCs *in vitro*, such as IL-10, VEGF-A, and HGF (Aggarwal and Pittenger, 2005). In particular, HGF promotes hepatic regeneration and exerts anti-fibrotic effects by enhancing hepatocyte proliferation and inhibiting apoptosis (Lee et al., 2018). It has been demonstrated that the stable expression of HGF in BM-MSCs improved cell homing capacity and differentiation into liver cells, thus alleviating CCl_4-induced liver fibrosis in rats (Sun et al., 2020). Therefore, the release of HGF in the parenchyma of damaged livers could be associated with the ability of MSCs to reverse the progression of liver fibrosis.

The MSC secretome has been proven to be safer and equally effective in liver regeneration (Driscoll and Patel, 2019). In fact, the secretome obtained by UC-MSCs, either undifferentiated or committed into hepatocyte-like cells, improved hepatic fibrosis both *in vivo* and *in vitro*. In particular, the UC-MSC secretome was enriched in the milk factor globule EGF 8 (MFGE8), an anti-fibrotic protein, which expression is reduced in fibrotic or cirrhotic livers. The MFGE8-containing secretome reduced the ECM deposition and suppressed the activation of HSCs by downregulating α-SMA expression and the TGF-β signaling pathway (An et al., 2017). A similar anti-fibrotic effect was also observed *in vitro* on TGF-β-activated HSCs, using conditioned medium derived from AMSCs (Fu et al., 2018) and from BM-MSCs (Huang et al., 2016). Interestingly, in a CCl_4-induced fibrosis murine model, the BM-MSC secretome also showed immunosuppressive properties, by reducing inflammatory infiltration, and pro-regenerative effects, by enhancing hepatocyte proliferation and promoting HSC apoptosis (Huang et al., 2016). Taken together, these findings suggest that the anti-fibrogenic effect of MSCs can be mediated through the release of paracrine factors, which include soluble factors and EVs.

Extracellular vesicles are small membrane particles delimited by a lipid bilayer membrane that are secreted by virtually all cells into the extracellular microenvironment and can be isolated from all biological fluids. EVs display great heterogeneity in size and molecular cargo, and since specific markers to distinguish one vesicle subpopulation from another are still lacking, EVs are currently classified based on their size and biogenesis in exosomes, ectosomes, and apoptotic bodies (Meldolesi, 2018; Bruno et al., 2020a).

Exosomes (30–120 nm) are small EVs that arise from the inward invagination of the membrane of endosomal structures, known as MVBs. Exosomes are released into the extracellular space upon fusion between MVBs and the plasma membrane. This process, called exocytosis, is principally coordinated by the ESCRT machinery (Meldolesi, 2018) and by other ESCRT-associated proteins, such as ALIX that contributes to cargo packaging into vesicles and triggers exosome formation (Baietti et al., 2012; Hurley and Odorizzi, 2012). Besides, an ESCRT-independent mechanism has been described in exosome formation and secretion (Stuffers et al., 2009). Among the proteins participating in this process, the tetraspanins CD63, CD81, and CD9 coordinate the specific sorting of cargo into exosomes (Chairoungdua et al., 2010; Nazarenko et al., 2010; van Niel et al., 2011; Perez-Hernandez et al., 2013), whereas the Rab GTPases Rab11, 27a, 27b, and 35 are involved in vesicle budding, transport along the cytoskeleton, and fusion with the plasma membrane (Savina et al., 2005; Stenmark, 2009; Hsu et al., 2010; Ostrowski et al., 2010; Bobrie et al., 2012; Zhen and Stenmark, 2015). Furthermore, exosome release can be controlled by calcium signaling (Savina et al., 2003), cytoskeleton

rearrangements (Granger et al., 2014), and ceramide synthesis (Trajkovic et al., 2008).

Extracellular vesicles generated by the direct outward budding of the plasma membrane are defined as ectosomes or microvesicles (100–1,000 nm). The modifications in the plasma membrane curvature result from changes in protein interactions, which involve the ARRDC1 and the late endosomal protein TSG101 (Nabhan et al., 2012), and from the calcium-dependent activation of enzymes, such as flippases, floppases, scramblases, and calpain, which alter the lipidic composition of the plasma membrane (van Niel et al., 2018). As for exosomes, the process of ectosomes vesiculation and release also relies on cytoskeleton rearrangements, controlled by the ROCK (Li et al., 2012) and the signaling cascade of Ras-related GTPase ARF6 (Muralidharan-Chari et al., 2009). Lastly, fragments of dead cells that are released through the plasma membrane blebbing of apoptotic cells are defined as apoptotic bodies (1,000–5,000 nm) (Hristov et al., 2004).

At present, EVs are considered as important mediators of intercellular communication (Lee et al., 2011; Lawson et al., 2017). In fact, the EV cargo includes proteins, lipids, and nucleic acids that can be transferred between cells protected from degradation, thus eliciting their intracellular uptake by endocytosis. The specific molecular content of EVs usually depends on the tissue of origin, but it can also be genetically modified to allow EVs to carry the desired therapeutic molecules (Varderidou-Minasian and Lorenowicz, 2020). Once internalized by target cells, EVs can modulate a number of physiological and pathophysiological processes, including metabolism, immune responses, tumor progression, and metastasis (Lou et al., 2017a).

MSC-EV EFFECTS IN LIVER FIBROSIS

Recently, the role of EVs in tissue repair and regeneration has been extensively studied. In particular, MSC-derived EVs have shown a pro-regenerative effect in several tissues, including heart, lung, bone, skin, brain, kidney, and liver (Fiore et al., 2018; Varderidou-Minasian and Lorenowicz, 2020). As reported for MSCs, also MSC-EVs exhibit therapeutic effects in several preclinical models of hepatic fibrosis (**Table 2**). In a context of liver fibrosis, MSC-EVs act by modulating different molecular pathways into hepatocytes, activated HSCs and immune cells (**Figure 1**).

The first evidence that MSC-EVs were able to alleviate liver fibrosis came from Li et al. (2013). In a mouse model of CCl_4-induced liver fibrosis, they showed that EVs derived from UC-MSCs ameliorated hepatic inflammation and collagen deposition. Moreover, *in vitro* UC-MSC-EV administration suppressed both the EMT and the TGF-β/SMAD signaling pathway through the inhibition of SMAD2 phosphorylation and the reduction of type I and III collagen and TGF-β transcripts expression in hepatocytes. The therapeutic effect of UC-MSC-EVs has been also demonstrated in schistosomiasis, a parasitic disease that leads to serious chronic liver inflammation (Dong et al., 2020). *In vivo* administration of UC-MSC-EVs in *Schistosoma japonicum*-infected mice alleviated hepatic fibrosis by downregulating the expression of α-SMA and types I and III collagen. The consequent inhibition of HSC activation was also confirmed *in vitro* on HSCs. In addition, UC-MSC-EVs reduced the mRNA expression of pro-inflammatory cytokines TNF-α, IL-1β, and IFN-gamma in schistosome-infected liver tissue. Also, EVs obtained from UC-PVCs can effectively reduce both fibrosis and inflammation in a TAA-induced model of chronic liver injury (Fiore et al., 2020). Interestingly, EVs derived from UC-PVCs that were transduced by an adenovirus vector to produce human IGF-1 exhibited a stronger anti-fibrotic effect with respect to their green fluorescent protein (GFP)-transfected counterpart. *In vitro* experiments demonstrated that treatment with IGF-1-containing UC-PVC-EVs reduced the activation of HSCs by downregulating the expression of type I collagen, α-SMA, and TGF-β1. Furthermore, IGF-1-containing UC-PVC-EVs converted pro-fibrogenic hepatic macrophages into anti-inflammatory phagocytes by increasing arginase-1 and downregulating iNOS, TNF-α, and IL-6 expression levels.

Another embryonic-derived source of MSCs is the amnion. In two different models of CLD, Ohara et al. (2018) have demonstrated the anti-inflammatory and anti-fibrotic effects of EVs obtained from AMSCs. In a rat model of NASH, induced by a HFD, AMSC-EVs downregulated the expression of pro-inflammatory cytokines TNF-α, IL-1β, and IL-6 and reduced the activation of pro-inflammatory M1 macrophages (Kupffer cells) in liver tissue. In CCl_4-induced hepatic fibrosis, treatment with AMSCs attenuated fibrosis by reducing the expression of α-SMA and the number of Kupffer cells. *In vitro*, AMSCs treatment reduced the expression of TNF-α in both HSCs and Kupffer cells activated by LPS and reduced the NF-kB transcriptional activity induced by LPS, through the inhibition of the phosphorylation of IkB-α and p65. Since AMSC-EVs did not affect NF-kB transcriptional activity induced by TRAF, one could speculate that AMSC-EVs might suppress the earlier steps of the LPS/TLR4 signaling pathway. Interestingly, another research study on a rat model of CCl_4-induced liver fibrosis demonstrated that EVs released by CP-MSCs were able to transfer miR-125b between MSCs and HSCs. The inhibition of miR-125b targets smo, resulting in the suppression of Hedgehog signaling with consequent amelioration of hepatic fibrosis (Hyun et al., 2015).

Recently, ESCs have been identified as an alternative source of MSCs. In a rat model of TAA-induced chronic liver injury, (Mardpour et al., 2018) reported the hepatoprotective effect of EVs obtained from human ESC-derived MSCs. In TAA animals, the ESC-MSC-EVs improved hepatocyte viability and reduced both apoptosis and the expression of pro-fibrotic molecules, such as collagen, α-SMA, and TIMP-1, while increasing the expression of collagenases, such as matrix metalloproteinase MMP-9 and -13. In addition, ESC-MSC-EVs exhibited immunomodulatory properties by reducing immune cell infiltration and modulating the expression of inflammatory cytokines, with a decrease in TNF-α and IL-2 levels and an increase in TGF-β and IL-10 levels. Interestingly, the anti-inflammatory effect of the ESC-MSC-EVs resulted to be stronger than the one of other somatic tissue-derived MSC secretome, such as BM-MSCs and ADSCs (Mardpour et al., 2018). However, the efficiency of EV treatments might

TABLE 2 | Anti-fibrotic effect of MSC-EVs in different models of chronic liver injury.

Source	Isolation method	Liver fibrosis model	Mechanism of action	References
Human UC-MSCs	Differential U.C. Concentration (100 kDa) Density gradient U.C. Filtration (0.22 μm)	CCl4 mouse model. HL7702 human epithelioid liver cell line activated by TGF-β1	Downregulation of fibrotic genes (COL1 and COL3), inactivation of the TGF-β/SMAD pathway (TGF-β1, SMAD2) and of EMT in hepatocytes (E-cadherin, N-cadherin, vimentin)	Li et al., 2013
Human UC-MSCs	Filtration (0.1 μm). Concentration (100 kDa) Density gradient U.C. Filtration (0.22 μm)	Schistosomiasis mouse model. LX2 activated by TGF-β1	Increased mice survival, improvement of liver function by downregulating pro-fibrotic genes (COL1, COL3, and α-SMA) and pro-inflammatory cytokines (TNF-α, IL-1β, and IFN-gamma)	Dong et al., 2020
Human UC-PVCs engineered to produce IGF-1	Differential U.C.	TAA mouse model	Downregulation of pro-fibrotic genes (COL1A2, α-SMA, and TGF-β)	Fiore et al., 2020
Human AMSCs	Differential U.C. Filtration (0.22 μm)	CCl4 rat model. NASH (HFD) rat model. Rat HSC and Kupffer cells activated by LPS	Reduction of Kupffer cell number and HSC activation, by downregulating pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) and pro-fibrotic genes (TGF-β1, α-SMA, and TIMP-1) and by inactivating the LPS/TLR4 signaling pathway (p65 and IkB-α phosphorylation) in HSCs and in Kupffer cells	Ohara et al., 2018
Human CP-MSCs	Differential U.C.	CCl4 mouse model. FBS-activated LX-2	Reduction in the expression of miR-125b target gene Smo resulted in the downregulation of the Gli family (downstream signaling molecules of Smo) and of the hedgehog signaling pathway in HSC	Hyun et al., 2015
Human ESC-MSCs	Differential U.C.	TAA rat model. Primary hepatocytes	Reduction of fibrosis and immune cell infiltration by upregulating collagenases (MMP-9 and MMP-13) and anti-inflammatory (TGF-β and IL-10) and anti-apoptotic (BCL-2) genes and by downregulating pro-fibrotic (COL1-α, α-SMA, and TIMP-1), pro-apoptotic (caspase-3, BAX), and pro-inflammatory genes (TNF-α and IL-2)	Mardpour et al., 2018
Human ESC-MSCs	Differential U.C.	TAA rat model	Reduction of necrosis, inflammation, and fibrosis by upregulating collagenases (MMP-9 and MMP-13) and anti-inflammatory (IL-10) genes and by downregulating pro-fibrotic (COL1-α, α-SMA, and TIMP-1), pro-apoptotic (cleaved caspase-3, BAX), and pro-inflammatory genes (TNF-α and IL-2)	Mardpour et al., 2019
miR-122-modified murine ADSCs	ExoQuick-TC kit	*In vitro* culture-induced LX-2 activation	Reduction of HSC proliferation and inhibition of the expression of COL1A1 and of miR-122 target genes: CCNG1, IGF1R, and P4HA1	Lou et al., 2017b
miR-181-5p-modified murine BM-MSCs	ExoQuick-TC kit	CCl4 mouse model. HST-T6 cells activated by TGF-β1	Amelioration of liver function, activation of autophagy by upregulating P62 and Beclin1, attenuation of fibrosis and inflammation by downregulating fibrotic genes COL1, COL3 vimentin, α-SMA fibronectin, of miR-181-5p targets Stat3 and Bcl-2, and pro-inflammatory cytokines (TNF-α, IL-6, and IL-17)	Qu et al., 2017
Murine BM-MSCs engineered with miR-223	Differential U.C.	Autoimmune hepatitis mouse model. Murine hepatocytes (AML12) treated with LPS and ATP	Improvement of liver structure and function, reduction of lymphocyte infiltration, with downregulation of pro-inflammatory cytokines (TNF-α, IL-1β, and IL-17A) and of pro-apoptotic proteins NLRP3 and caspase-1	Chen et al., 2018
Human BM-MSCs	Differential U.C.	CCl4 rat model. Activated HSCs	Improvement of liver function and reduction of fibrosis, inflammation and HSC activation through the inhibition of fibrosis-related proteins (COL1, α-SMA) and of the Wnt/β-catenin pathway (PPAR-gamma, β-catenin, Wnt3a, Wnt10b, WISP1, cyclin D1)	Rong et al., 2019
HLSCs	Differential U.C. Filtration (0.22 μm)	NASH (MCDD) mouse model	Improvement of liver function and reduction of fibrosis and inflammation by increasing the anti-inflammatory cytokine IL-10 and by downregulating fibrosis-associated genes (α-SMA, COL1-α1, TGF-β1, and Ltbp1), genes involved in tissue remodeling and in inflammation, such as TIMP-1; MMP-1a, -13, -14, and -8; IFN-γ; TNF-α; and IL-1β	Bruno et al., 2020b

be reduced by a rapid clearance of EVs from the target organ. To extend the bioavailability of the EVs in the liver, the use of ESC-MSC-EVs encapsulated in polyethylene glycol macromeres has been proven to be effective in a TAA rat model of hepatic fibrosis. Once injected into the peritoneum cavity, the hydrogel-released EVs were gradually swollen upon biodegradation and progressively released over 1 month, resulting in liver accumulation. Histological and molecular analysis have pointed out that, compared with freely injected EVs, the hydrogel-released EVs had stronger anti-fibrotic, anti-apoptotic, and anti-inflammatory effects in fibrotic liver tissue (Mathieu et al., 2019).

Adipose tissue-derived MSC have also shown therapeutic effects in hepatic fibrosis (Lou et al., 2017b; Qu et al., 2017). Qu et al. (2017) demonstrated that the transient over-expression of miR-181-5p in ADSCs increased their therapeutic potential in a

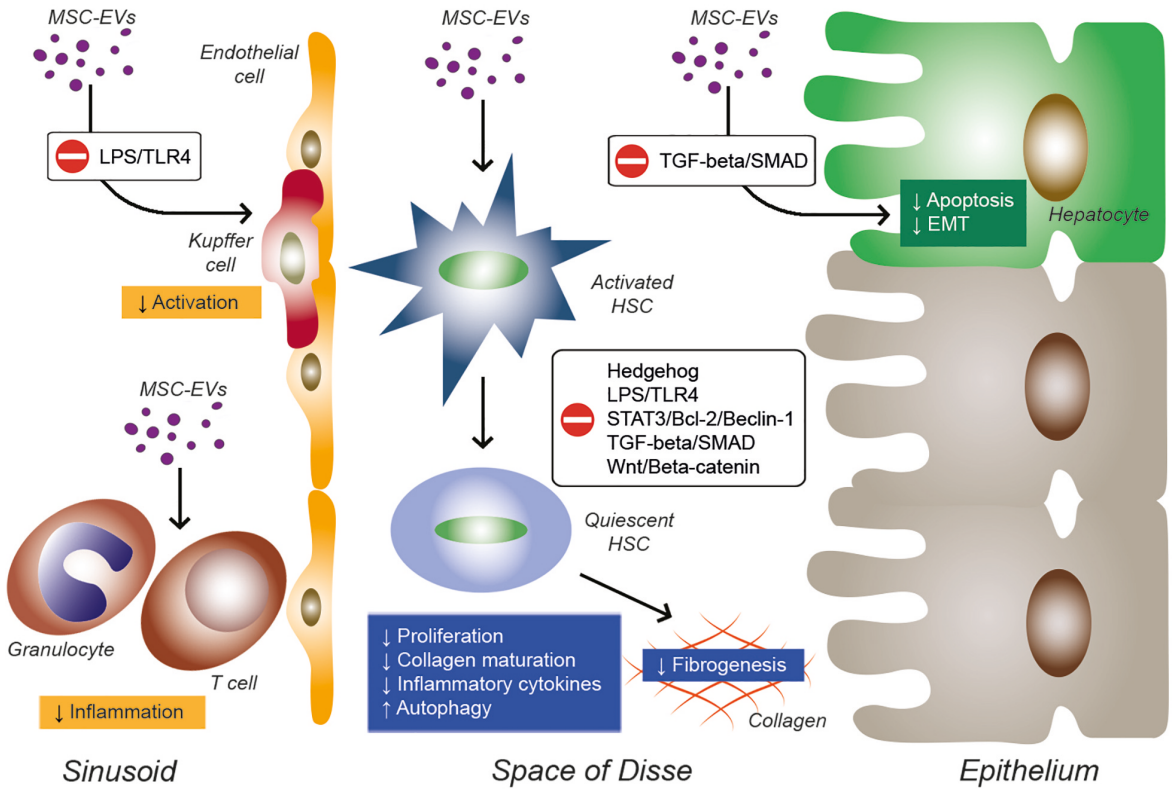

FIGURE 1 | Effects of EVs derived from MSCs on inflammatory cells, Kupffer cells, hepatic stellate cells (HSCs), and hepatocytes during liver fibrosis. EVs from MSCs derived from different sources can modulate various molecular pathways in target cells by reducing inflammation, Kupffer cell activation, HSC activation and fibrogenesis, and hepatocyte apoptosis and EMT.

CCl_4-induced fibrosis model. In addition, the miR-181-5p over-expressing ADSC-EVs induced autophagy in HSCs by inhibiting the STAT3/Bcl-2/Beclin pathway and suppressed HSC activation induced by TGF-β through the reduction of fibrosis-related genes, such as fibronectin and type I and III collagen (Qu et al., 2017). Another study showed that the engineering of ADSCs with a miR-122 over-expressing lentiviral vector made the ADSC-EVs more effective against liver fibrosis than their non-transfected counterpart. In fact, ADSC-EVs efficiently transfer miR-122 to HSCs, inhibiting cell cycle progression and suppressing miR-122-target genes CCNG1, IGF1R, and P4HA1, which regulate proliferation and collagen maturation in HSCs (Lou et al., 2017b).

Extracellular vesicle engineering has been proven effective against liver fibrosis also for MSC-EVs derived from other sources, such as the bone marrow. In a murine model of autoimmune hepatitis, EVs derived from BM-MSCs improved liver function by downregulating the expression of inflammasome and apoptosis-related genes NLRP3 and caspase-1. The lentivirus-driven upregulation of miR-223 in BM-MSCs improved the anti-inflammatory and cytoprotective properties of BM-MSC-EVs, both *in vitro* and *in vivo*, whereas the specific inhibition of miR-223 completely abrogated the therapeutic effect of BM-MSC-EVs (Chen et al., 2018). Besides, the administration of BM-MSC-EVs in CCl_4-induced liver fibrosis suppressed the Wnt signaling pathway in activated HSCs by downregulating the expression of β-catenin, Wnt3a, Wnt10b, and PPAR-gamma (Rong et al., 2019).

Recently, our group has investigated the therapeutic effect of EVs released by HLSCs in CLD (Bruno et al., 2020b). HLSCs are stem cell populations resident in human adult liver that have MSC-like features (phenotype, gene expression profile, multilineage differentiation, and immunoregulatory capacities). Moreover, HLSCs showed commitment toward hepatic lineage and contributed to tissue regeneration in different experimental animal models of liver injury (Herrera et al., 2006; Bruno et al., 2019). In a murine model of liver fibrosis, HLSC-EVs improved liver function and morphology through the reduction of fibrosis and inflammation. Molecular analyses revealed that HLSC-EV administration downregulated most of the fibrosis-associated genes, such as type I collagen, α-SMA, TGF-β, and the gene latent-TGF-β-binding protein 1 (Ltbp1), which expression was increased by MCDD. The HLSC-EVs treatment also reverted the expression of genes involved in tissue remodeling (MMP-1a, -8, -13, and -14 and TIMP-1) and in inflammation (IFN-gamma, IL-1β, and TNF-α). Furthermore, the increase of IL-10 expression levels and the reduction of inflammatory infiltrations in the liver confirmed the anti-inflammatory potential of HLSC-EVs. Interestingly, proteomic analyses of HLSC-EV cargo revealed a number of anti-inflammatory proteins that might contribute to the improvement of liver fibrosis.

CONCLUSION

Mesenchymal stromal cells treatment can reduce fibrosis by downregulating gene expression levels of different transcripts fundamental for fibrosis development, such as α-SMA, TGF-β, and collagens. In liver, the regression of fibrosis could be also attributed to the enhanced levels of MMPs secreted by MSCs that degrade and remodel the fibrotic matrix. Immunomodulatory capacities of MSCs could also be implicated in anti-fibrotic effects by coordinating the recruitment and the polarization of inflammatory cells and the production of cytokines, thus regulating the pro-fibrotic environment. However, it is still debated whether MSCs could engraft into the liver and whether, once engrafted, MSCs could differentiate into hepatocytes, thus participating in liver regeneration, or into pro-fibrotic cells, thus getting worse both liver function and morphology. To avoid the differentiation of MSCs into pro-fibrotic cells, the use of the MSC secretome would be preferable over the MSC treatment. Several research articles indicated that secretome could mimic the effects of cell injection and exert similar anti-fibrotic effects. Among the paracrine factors produced by MSCs, EVs emerge as a valid and alternative tool to cell treatment. Compared with cell-based approach, the EV treatment shows some benefits, such as the possibility of avoiding cell misdifferentiation and the consequent aggravation of liver fibrosis. In fact, to our knowledge, no evidence of fibrogenic potential of MSC-EVs has been reported. In addition, the use of MSC-EVs has a higher efficacy profile than MSC therapy since, once injected *in vivo*, the EVs pass through the biological barriers and shuttle different molecules (proteins, RNAs, and lipids) to target cells and tissues. Interestingly, in the context of CLD, MSC-EVs have been proven to regulate different molecular pathways in liver cells, such as the TGF-β/SMAD, the LPS/TLR4, the STAT3/Bcl-2/Beclin, and the Wnt signaling pathway.

Despite the promising results obtained in CLD preclinical studies with MSC-EVs, many problems need to be solved prior to clinical EV applications. Currently, EV purification is a challenging topic: several protocols have been proposed for EV isolation, and most of them can be combined to achieve higher purity of the EV sample by removing cell debris, protein aggregates, and vesicles of non-endosomal origin, such as lipoproteins (Mathieu et al., 2019). Differential ultracentrifugation represents the "gold standard" for EV purification (Théry et al., 2006), but long times, possible EV damage and contamination by non-EV particles hamper the clinical application of this technology. An alternative approach to isolate distinct EV subtypes is density gradient centrifugation, which consists in an iodixanol gradient able to separate different EV populations based on their density (Iwai et al., 2016). Moreover, the density gradient ultracentrifugation may be combined with floating to separate vesicles from contaminant proteins (Kowal et al., 2016). Immunoaffinity capture technology takes advantage of EV-associated molecules, such as tetraspanins and ESCRT proteins, but the lack of specific EV subpopulation markers makes it difficult to distinguish one type of vesicle from another (Lozano-Andrés et al., 2019). The majority of these techniques are suitable for research but limited to application for scalable production of EVs for therapeutic purposes. Membrane filtration is potentially applicable to large volumes of conditioned medium. This technique has been proven to be effective in separating EV subpopulations based on their density and hydrodynamic properties (Heinemann et al., 2014). Tangential flow filtration has been also proposed for scalable production of EVs from large volumes of conditioned medium (Busatto et al., 2018). Similarly, size exclusion chromatography has revealed one of the most rapid EV isolation techniques, particularly indicated for exosome purification (Blans et al., 2017). Even if EVs appeared more stable and resistant for long-term storage than MSCs, further studies on pharmacological and pharmacodynamic properties are needed to clarify whether the use of MSC-EVs is suitable for clinical application.

AUTHOR CONTRIBUTIONS

SB, GCh, and CP performed the research of the pertinent literature and designed and drafted the manuscript. GCa revised and edited the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Molecular Crosstalk Between Macrophages and Mesenchymal Stromal Cells

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Mesenchymal stromal cells (MSCs) have been widely investigated for regenerative medicine applications, from treating various inflammatory diseases as a cell therapy to generating engineered tissue constructs. Numerous studies have evaluated the potential effects of MSCs following therapeutic administration. By responding to their surrounding microenvironment, MSCs may mediate immunomodulatory effects through various mechanisms that directly (i.e., contact-dependent) or indirectly (i.e., paracrine activity) alter the physiology of endogenous cells in various disease pathologies. More specifically, a pivotal crosstalk between MSCs and tissue-resident macrophages and monocytes (TMφ) has been elucidated using *in vitro* and *in vivo* preclinical studies. An improved understanding of this crosstalk could help elucidate potential mechanisms of action (MOAs) of therapeutically administered MSCs. TMφ, by nature of their remarkable functional plasticity and prevalence within the body, are uniquely positioned as critical modulators of the immune system – not only in maintaining homeostasis but also during pathogenesis. This has prompted further exploration into the cellular and molecular alterations to TMφ mediated by MSCs. *In vitro* assays and *in vivo* preclinical trials have identified key interactions mediated by MSCs that polarize the responses of TMφ from a pro-inflammatory (i.e., classical activation) to a more anti-inflammatory/reparative (i.e., alternative activation) phenotype and function. In this review, we describe physiological and pathological TMφ functions in response to various stimuli and discuss the evidence that suggest specific mechanisms through which MSCs may modulate TMφ phenotypes and functions, including paracrine interactions (e.g., secretome and extracellular vesicles), nanotube-mediated intercellular exchange, bioenergetics, and engulfment by macrophages. Continued efforts to elucidate this pivotal crosstalk may offer an improved understanding of the immunomodulatory capacity of MSCs and inform the development and testing of potential MOAs to support the therapeutic use of MSCs and MSC-derived products in various diseases.

Keywords: macrophages (M1/M2), mechanism of action (MOA), immunomodulation, cell therapy, mesenchymal stromal (or stem) cells

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INTRODUCTION

Mesenchymal stromal cells (MSCs), also referred to as Mesenchymal Stem Cells or Medicinal Signaling Cells, have garnered attention as cell therapies against various diseases, including graft versus host disease (GvHD) (Ball et al., 2013), neurological disorders (Riordan et al., 2019; Sun et al., 2020), and cardiovascular disease (Hare et al., 2009). For decades, MSCs have been investigated for their immunomodulatory, anti-inflammatory, and regenerative functions revealing several potential modalities for mediating therapeutic effects. A large body of literature has demonstrated that MSCs are highly responsive to environmental cues and elicit their effects through direct [i.e., cell–cell contact (Quaedackers et al., 2009; Galipeau and Sensebe, 2018)] and indirect (i.e., paracrine signaling (Caplan and Correa, 2011; Salgado and Gimble, 2013; Serejo et al., 2019)] interactions resulting in suppression of pathogenic cells (Ren et al., 2008; Quaedackers et al., 2009), induction of regulatory cells (Luz-Crawford et al., 2013; Lee et al., 2017), cytoprotection (Block et al., 2009), trophic support (Zhang et al., 2007), and tissue repair (Wu et al., 2007). Thus, the therapeutic capacity of exogenously administered MSCs relies on their innate ability to respond to surrounding pathophysiological cues and orchestrate cellular and molecular changes to restore local and systemic homeostasis.

Although MSCs hold promise for clinical use, our knowledge of MSCs has been gained primarily through *in vitro* assays and pre-clinical animal studies, leaving gaps in translation and an inability to demonstrate definitive efficacy in human clinical trials (Galipeau and Sensebe, 2018). How we characterize MSCs and test their therapeutic "potencies" *ex vivo* may be the reason for these disparities that are observed between preclinical and clinical trial results. Further elucidations into cellular and molecular interactions mediated by MSCs will better inform future investigations of key endogenous cellular targets and, ultimately, bridge the gaps to advance clinical use of MSCs by understanding how, when, and where to deliver therapeutic MSCs.

MSCs have been isolated from various tissues of the body including bone marrow, adipose, and umbilical cord tissue. By harnessing the plastic-adherence property of MSCs and with the addition of a tailored media formulation for sustaining their growth, the residual tissue-resident cells can be eliminated and MSCs can be obtained for investigational use (Secunda et al., 2015; Palumbo et al., 2018). Culture systems enable expansion of MSCs for acquiring the necessary cell numbers (i.e., yield) for therapeutic dosages in humans or animal studies. Regardless of the tissue source, plastic-adherent MSCs are further characterized by a specific set of criteria such as their expression profile of positive (e.g., CD73, CD90, and CD105) and negative (e.g., CD11b, CD14, CD19, CD34, CD45, CD79a, and HLA-DR) surface markers and *in vitro* multi-lineage differentiation capacity (i.e., induced osteogenesis, adipogenesis, and chondrogenesis) (Dominici et al., 2006). The caveat, however, is that *ex vivo* manipulation to isolate, expand, and interrogate MSCs may introduce transcriptional, epigenetic, metabolomic, and proteomic changes – and these characteristics of cultured MSCs likely do not parallel those of endogenous stromal cells (Caplan, 2008). Moreover, delivery of therapeutic MSCs back to an *in vivo* environment makes it additionally challenging to anticipate outcomes and demonstrate reproducible results. For these reasons, we will primarily focus on MSCs infused as therapy and the consequential effects on endogenous cells.

Autologous or allogeneic MSCs delivered as therapy may exert multiple effects to mitigate local and systemic pathologies. These potential therapeutic modalities are a result of the dynamic ability of MSCs to respond to various stimuli (Caplan and Correa, 2011). The caveat, however, is that these multimodal effects of MSCs make it challenging to identify specific mechanisms of action (MOA) that, if realized, can then be exploited in developed testing platforms. In fact, there is a strong need to develop and test a multivariate set of assays to evaluate mechanistic outcomes of MSCs using co-cultures with immune cells. Not only would understanding biological variation, evaluating manufacturing processes, and evaluating tissue sources be improved, these assays would help predict *in vivo* "therapeutic potencies" of infused MSCs, ultimately facilitating the translation and standardization necessary to advance cell manufacturing and regulatory approval for clinical use (Bianco et al., 2008; Chinnadurai et al., 2018). *In vitro* and *in vivo* preclinical studies have, thus far, provided compelling evidence for key interactions, referred to as molecular crosstalk, between MSCs and immune cells, most notably monocytes, macrophages, and T lymphocytes (Bianco et al., 2008; Chinnadurai et al., 2018; Antebi et al., 2019). These vital immune cells are at the crux of immune system functions, transmitting information in the form of molecular signals from a site of pathology to the rest of the body. Here, we take a more in-depth look into evidence that suggests an integral crosstalk between MSCs and specifically monocytes and tissue-resident macrophages (TMφ) to get us one step closer to identifying potential MOAs by MSCs.

CHANGING DOGMA DELINEATE MONOCYTE AND MACROPHAGE POPULATIONS

First, understanding the physiological roles of TMφ during steady-state (i.e., homeostasis) and pathology is necessary to realize the alterations mediated by MSCs, and vice versa. Monocytes and macrophages, as well as dendritic cells, comprise the mononuclear phagocytic system (MPS). More in-depth physiological roles will be further described herein, but the most simplified term that captures the functions of MPS cells is "SHIP" – Sample, Heal, Inhibit, and Present (antigen) (Mills et al., 2014). Together, MPS cells are essential cells of the innate immune system that acquire information from their surroundings (e.g., by phagocytosis) and communicate the information (e.g., by antigen-presentation) to the adaptive immune system for a coordinated resolution of pathology. Thus, physiological plasticity (i.e., functional heterogeneity) is the underlying propensity of these cells to regulate tissue microenvironments (Wynn et al., 2013). With the dynamic

nature of both MSCs and TMφ, determining the mechanistic effects resulting from this integral crosstalk continues to be an ongoing exploration.

The literature depicting the physiology of macrophages often pays homage to the pioneering work of Ilya Metchnikoff, a Russian zoologist turned immunologist and Nobel Prize laureate. He not only described these "large devouring cells" in the late 19th century but, more importantly, suggested the role of these cells as part of the host's defenses, the first implications of the innate immune system, deeming him the father of cellular immunity (Hoeffel and Ginhoux, 2015). Discoveries after that showed commonalities in cellular responses and phagocytic functions between bone marrow-derived monocytes and tissue-resident macrophages that led many to believe that monocytes were the predecessors of macrophages. Although these physiological similarities are still appreciated, fate mapping and lineage tracing technologies have more recently delineated their ontogenies. Hematopoiesis in the bone marrow generates myeloid precursors that differentiate into monocytes upon emigration to the bloodstream. TMφ can develop from circulating monocytes that have infiltrated into tissues. However, most of them originate from either yolk sac-derived erythro-myeloid or fetal liver progenitors during embryogenesis, i.e., hematopoietic stem cell-independent precursors (Dey et al., 2014; Hoeffel and Ginhoux, 2015). Further delineation of monocytes and macrophages describes differentiation into subsets and transitional phenotypes, respectively, with distinct functions influenced by spatiotemporal cues.

Monocytes are generally categorized into subsets corresponding to surface markers and functional activities, some of which are shared with TMφ. Interestingly, the frequency of each monocyte subset is inversely related to their lifespan during a steady state. Classical monocytes ($CD14^{high}CD16^{-}$) make up about 85% of the circulating monocytes, and 15% consist of both intermediate ($CD14^{high}CD16^{+}$) and non-classical monocytes ($CD14^{low}CD16^{high}$) (Patel et al., 2017; Ong et al., 2019). Classical monocytes are considered less mature and emerge from the bone marrow, where they enter the circulatory system with a propensity for phagocytosis of debris or foreign invaders, with the shortest lifespan of 1 day. The majority of these cells die or extravasate into tissues, whereas a small portion transition into intermediate monocytes. Intermediate monocytes are generated in response to an initial stimulus and function to propagate inflammatory signaling over a lifespan of a little over 4 days. Intermediate monocytes then transition to non-classical monocytes, with have an extended lifespan of approximately 7.5 days, allowing them to patrol the vasculature and potentially infiltrate affected tissues to resolve the inflammatory stimulus (Thomas et al., 2015; Patel et al., 2017). Thus, intermediate and non-classical monocytes are considered the mature "inflammatory" subsets as their frequency is elevated in the blood during inflammation or pathogenesis (Hamilton and Tak, 2009; Thomas et al., 2015). Although circulating monocytes have been observed to extravasate into tissues, especially during pathological activities, the majority provide short-term surveillance as a host's first line of defense and are then replenished by continued hematopoiesis (van Furth and Cohn, 1968).

In contrast, tissue-resident macrophages possess dynamic phenotypes and functions, some of which exhibit tissue-specific functions. Here, TMφ self-renew and persist for months to even years in steady-state. Upon activation by inflammation or other pathological stimuli, naïve macrophages (M0) differentiate into classical or alternative activation macrophages, formerly M1 or M2 phenotypes, respectively, according to their surrounding microenvironment. Initially, the M1 or M2 phenotypes denoted a pro- or anti-inflammatory function, respectively, and this paradigm was synonymous with the polarized responses of toll-like receptor (TLR) signaling observed with T lymphocyte subset (i.e., type 1 or 2 helper T cells) (Mills et al., 2000). Although this macrophage nomenclature is still used, emergent evidence now suggests a spectrum of spatiotemporal identifiers, ultimately relinquishing these finite conventions (Nahrendorf and Swirski, 2016). The dynamic phenotypes of the classical or alternative activation macrophage subsets have been identified to capture physiological functions closely correlated to metabolic programs (Mills et al., 2000; Vasandan et al., 2016). A burgeoning research area endeavors to ascertain their spatiotemporal role, however, this review will collectively consider macrophages regardless of the nomenclatures describing subsets, phenotypes, and tissue-specific names to describe their crosstalk with MSCs (**Table 1**).

FUNCTIONAL HETEROGENEITY OF MACROPHAGES

TMφ are vital cells of the body that are integral to the development and homeostatic maintenance of all tissues (**Figure 1**). These professional phagocytes have shared roles and responses across tissues, including clearance of dead cells and debris, presenting antigen, remodeling of tissue, and metabolic regulation but also functions that serve tissue-specific demands related to each organ. Interestingly, some physiological functions enable others. For instance, one of the primary TMφ of the bone, osteoclasts, are responsible for bone resorption which is fundamental to bone remodeling during development; thus, osteoclasts sculpt the bone cavity, enabling hematopoiesis to ensue. Consequently, the generation of monocytes and subsequently immune system functions rely on these bone resident TMφ. Throughout the body, these shared roles act specifically to maintain steady state functions of resident cells and organ system functions.

During erythropoiesis, macrophages surround maturing erythroblasts, ingest extruding nuclei and essentially permit formation of erythrocytes, or red blood cells. The depletion of erythrocytes for natural turnover is also a steady state function of splenic and hepatic TMφ. The liver, pancreas, and adipose tissue are organs that maintain metabolic homeostasis. TMφ of the liver, called Kupffer cells, facilitate the metabolism of hepatocytes during caloric intake, regulating the uptake, synthesis, and oxidation of fatty acids. Similarly, TMφ support β-cell function in the pancreas, although their precise role during steady state remains to be determined since discoveries so far appear to be a

TABLE 1 | Nomenclature used to denote monocyte and macrophage subsets with associated phenotypic markers and functions found in humans.

Nomenclature	Phenotypic markers	Function	Citation
Classical monocytes (Naïve)	HLA-DR^{+}, CD11b^{+}, CD14^{high}, CD16^{-}, CCR2^{high}, CXC3R1^{low}	Phagocytosis	Ong et al., 2019; Thomas et al., 2015
Intermediate monocytes (activated)	HLA-DR^{+}, CD11b^{+}, CD14^{high}, CD16^{+}, CCR2^{+}, CXC3R1^{high} CCR5^{+}	Pro-inflammatory	Thomas et al., 2015; Ong et al., 2019
Non-classical monocytes (activated)	HLA-DR^{+}, CD11b^{+}, CD14^{low}CD16^{high}, CCR2^{low}, CX3CR1^{high}	Patrolling	Thomas et al., 2015; Ong et al., 2019
Classical activation (M1) TMφ	HLA-DR^{+}, CD68^{+}, CD80^{high}, CD206^{low} CD40^{+}, CCR7^{+}, CXCL9^{+}, IL-10^{low}, IL-12^{high}	Pro-inflammatory, microbicidal, Th1 differentiation, tumor resistance	Martinez et al., 2008; Raggi et al., 2017; Shapouri-Moghaddam et al., 2018
Alternative activation (M2) TMφ	HLA-DR^{+}, CD68^{+}, CD86^{+}, CD80^{low}, CD206^{high}, CD36^{+}	Anti-inflammatory, reparative	Martinez et al., 2008; Raggi et al., 2017; Shapouri-Moghaddam et al., 2018
M2a subset	CD206+, IL-1R^{+}, CCL17^{+}	Anti-inflammatory, promote cell growth, wound healing	Martinez et al., 2008; Shapouri-Moghaddam et al., 2018
M2b subset	CD86^{+}, CCL1^{+}, IL-10R^{+}, IL-12R^{+}, IL-6R^{+}, IL-10^{high}, IL-12^{low}	Immunoregulation, promote tumor progression, Th2 differentiation, microbial infections	Martinez et al., 2008; Shapouri-Moghaddam et al., 2018
M2c subset	CD206^{+}, CD163^{+}, CXCL13^{+}, TLR-1^{+}, TLR-8^{+}	Immunosuppression, phagocytosis, tissue remodeling	Martinez et al., 2008; Shapouri-Moghaddam et al., 2018
M2d subset	IL-10R, IL-12R, IL-10^{high}, IL-12^{low}, TNFα^{low}	Angiogenesis, tumor progression	Martinez et al., 2008; Shapouri-Moghaddam et al., 2018

Alternative activation (M2) TMφ are further delineated into four subsets (a–d) with specific markers and functions.

consequence of pancreatic dysfunction, such as insulin resistance. Insulin and other hormone sensitivities are maintained by TMφ in adipose tissue for systemic metabolic regulation and thermogenic control of the body (Hamilton and Tak, 2009; Wynn et al., 2013).

Microglia are the main brain-resident TMφ responsible for neuronal patterning, survival, and function while other TMφ in the brain are localized to key areas for maintaining fluid balance and the integrity of the blood-brain barrier (Chen et al., 2015). Understandably, TMφ are fundamental constituents of the heart, lungs, and other organ systems, playing key roles during development and throughout adulthood; however, it is mostly through inflammatory and pathological conditions that their roles are elucidated. It is of no surprise that TMφ are involved in almost every disease and crosstalk with neighboring stromal cells serves as a vital connection to restore homeostasis of tissues.

CELLULAR CROSSTALK WITH MSCs

Several of the proposed MOAs that have been implicated as the underlying therapeutic effects of administered MSCs directly or indirectly acts on TMφ (**Figure 2**). Initial pathogenesis or physical insult mount a "SOS" response, recruiting cells (e.g., TMφ) that are proximal and distal to the site of pathology by chemotactic and tropic factors. MSCs, too, have demonstrated an ability to home to sites of pathology once infused as therapy (Shi et al., 2007). However, the most common route of delivery for MSCs is currently by intravenous infusion, rendering MSCs lodged in the capillaries of the lungs soon after and no evidence of engraftment to date (Giri and Galipeau, 2020). This evidence has markedly shaped approaches to investigate and exploit alternative mechanisms that may be employed by MSCs to mediate therapeutic effects. We now recognize that the effects of MSCs following infusion could be largely paracrine mediated, and not by direct cell–cell contact (Caplan and Dennis, 2006; Caplan and Correa, 2011).

Soluble mediators may play more than one role in resolving tissue damage or pathology. These signals can be released directly into extracellular spaces to incite local responses or packaged in extracellular vesicles, e.g., exosomes, which travel to distal sites for systemic responses. The constituents of this cellular crosstalk described here are, in many cases, likely produced by MSCs that then directly influence the metabolic program and, in turn, the physiological function of TMφ. Alterations in macrophages are detected as skewed phenotypes as a result of metabolic reprogramming, although the duration of these temporal changes, downstream targets, and overall *in vivo* effects during pathology remain to be fully elucidated locally and systemically.

COX/PGE2/EP4 Axis

One of the most well-known soluble mediators that has been attributed to the therapeutic effects of MSCs is prostaglandin E2 (PGE2). PGE2 is a homeostatic factor derived from the metabolism of arachidonic acid by prostaglandin synthases and cyclooxygenases (constitutively active COX1 and inducible COX2) in both myeloid and stromal cells (Kalinski, 2012). Both human and mouse MSCs constitutively produce PGE2, and upon pro-inflammatory challenge with interferon-γ (IFNγ), tumor necrosis factor-α (TNFα) or interleukin (IL)-1β, induced elevation of PGE2 has been demonstrated (Noronha et al., 2019). PGE2 promotes the production of interleukin-10 (IL-10) from TMφ and has a synergistic effect with indoleamine 2,3-dioxygenase (IDO) to elicit MSC-induced

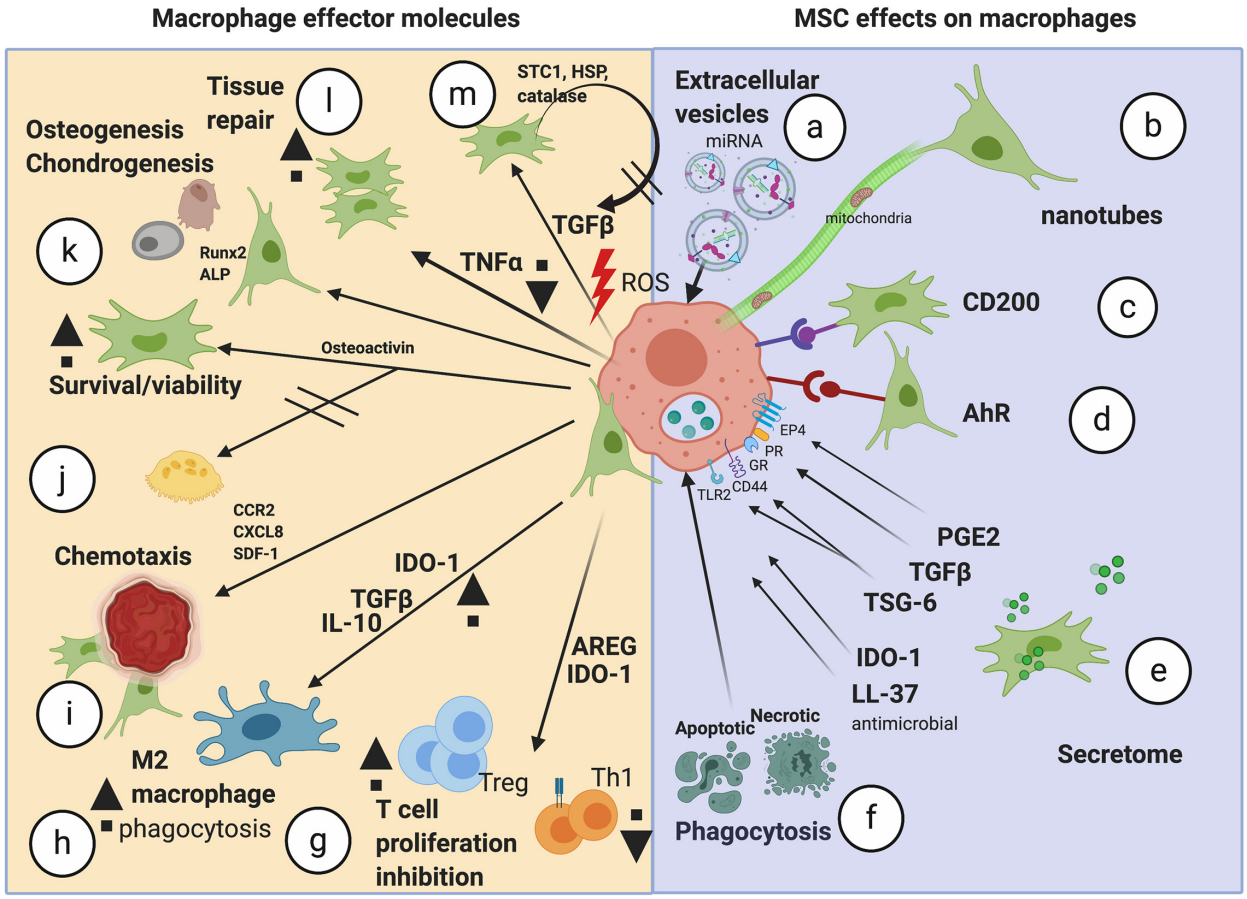

FIGURE 1 | Schematic of the potential mechanisms of action mediated by MSCs on TMφ. The range of mechanisms important in MSC effects on macrophages (a–f) and the resulting effector molecules/effects seen in macrophages (g–m). miRNA, microRNA; AhR, aryl hydrocarbon receptor; PGE2, prostaglandin E2; TGFβ, transforming growth factor beta; TSG-6, TNF alpha-stimulated gene 6; IDO-1, indoleamine 2,3 dioxygenase; LL-37, antimicrobial peptide; AREG, amphiregulin; Tregs, regulatory T cells; IL-10, interleukin-10; CCR2, monocyte chemotactic protein −1 receptor; CXCL8, chemokine C-X-C motif chemokine ligand 8; SDF-1, stromal cell derived factor 1; Runx2, Runt-related transcription factor 2; ALP, alkaline phosphatase; LPS, lipopolysaccharide; TNFα, tumor necrosis factor alpha; ROS, reactive oxygen species; HSP, heat shock protein; STC1, stanniocalcin-1; Image created with BioRender.

immunosuppression on various immune cells *in vitro* (Spaggiari et al., 2008; Nemeth et al., 2009). An MSC-dependent PGE2 has been demonstrated to alter monocyte-to-macrophage differentiation, promoting the survival of monocytes activated by macrophage colony stimulating factor (M-CSF), by *trans*-activation of the M-CSF receptor (Digiacomo et al., 2015) and, more importantly, polarized to an alternative activation (M2-like) phenotype (increased CD163 and CD206 and reduced MHCII/HLA-DR expressions) of TMφ (**Figure 3F**). Alternative activation macrophages upregulated secretion of amphiregulin (AREG) (Ko et al., 2020), showed bolstered functions of scavenging and phagocytic activities and enhanced production of immunomodulatory cytokines IL-10 and transforming growth factor-β (TGFβ) *in vitro* (Chiossone et al., 2016). MSCs from multiple tissues have reproducibly polarized macrophages of various sources by this PGE2-dependent mechanism resulting in the suppression of pro-inflammatory factors, e.g., tumor necrosis factor α (TNFα), IL-12p70, and IL-17, while promoting anti-inflammatory IL-10, ultimately inhibiting perpetuation of immune responses by antigen presentation (Melief et al., 2013; Deng et al., 2016; Manferdini et al., 2017). PGE2 binding to EP4 activates adenylate cyclase and intracellular cAMP levels are elevated. This in turn activates PKA and this has been shown to phosphorylate CREB (cyclic AMP-responsive element binding). Phosphorylated CREB leads to transcription of C/EBP-β which promotes anti-inflammatory gene expression (Na et al., 2015). Of all the PGE2 receptors, only EP4 was found to facilitate the production of IL-10 and suppression of TNFα (Yasui et al., 2015). Furthermore, the secreted molecules released by the M2-like macrophages induced regulatory T cells (Tregs), a T cell subset essential for immune tolerance (Schmidt et al., 2016).

Tregs and AREG

MSCs skew TMφ phenotype via the secretome, with the resulting pro-regenerative macrophages demonstrating heightened release of IL10 and TGFβ (Francois et al., 2012; Mittal et al., 2016; Vasandan et al., 2016). However, MSC-conditioned macrophages

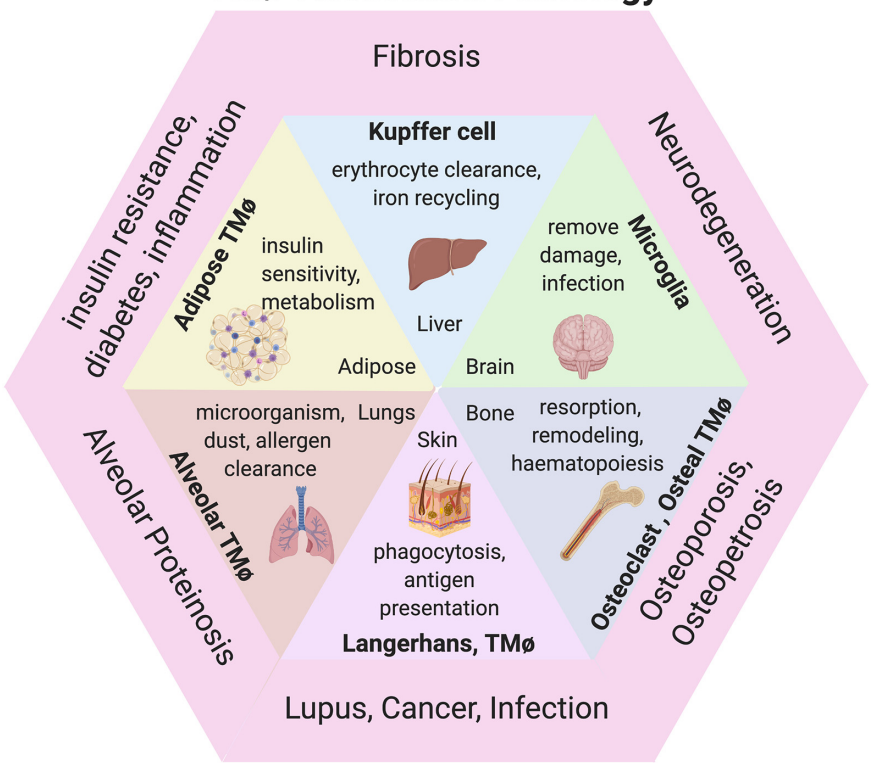

FIGURE 2 | TMφ-associated pathologies. Diagram of TMφ functions described during physiological (inside) and pathological presentations of disease (outer) corresponding to organs. Image created with BioRender.

are known to influence T cell activation *in vitro*, by inducing the differentiation of FoxP3^{+} Tregs from CD4+ helper T cells (Schmidt et al., 2016). Tregs are mediators of self-tolerance, essential to prevention of autoimmunity, and are immunosuppressive of inflammatory and allergic responses to infection. Tregs are recognized as significant contributors to immunomodulatory responses mediated by MSCs and in-depth descriptions of these interactions are described by Burr et al. (2013) and English (2013). MSC-primed macrophages, utilize TGFβ, IL-10 and other immunomodulatory factors that alter the differentiation of T cell subsets, dendritic cells and B cells *in vitro* as well as new *in vitro* and *in vivo* evidence points to epidermal growth factor receptor ligand AREG as another signaling moiety (Ko et al., 2020).

AREG has been implicated in the resolution of inflammation, regeneration of tissues, and restoration of homeostasis after injury. There are several ways that MSCs can promote the secretion of AREG from macrophages. These include the uptake of mitochondria from MSCs and the use of the COX-2/PGE2/EP4 signaling axis (Ko et al., 2020). In a mouse model of retinal inflammation, AREG suppressed immune responses by upregulating Tregs and downregulating Th1 cells. Recombinant AREG, administered alongside MSCs in macrophage depleted mice, showed some level of recovery of retinal pathology (Ko et al., 2020).

Metabolic Reprogramming of TMφ by MSCs

The metabolic profile of the macrophage could be a key determinant of phenotype and function. Steady state macrophages exhibit a metabolism that utilizes glucose and oxygen for mitochondrial oxidative phosphorylation to generate energy in the form of ATP. Stimulation toward the classical activation phenotype *in vitro* demonstrated that a metabolic switch to glycolysis facilitated pro-inflammatory functions *in vitro* (El Kasmi and Stenmark, 2015). Classical activation (M1-like) macrophages stimulated by lipopolysaccharide treatment *in vitro*, indicated an upregulation of 31 metabolic enzyme/transporter-related genes which confirmed increased glycolysis, the citric acid cycle intermediate succinate, and release of pro-inflammatory IL-1β. Succinate was inferred as a key metabolite that enhances pro-inflammatory signaling during inflammation (Tannahill et al., 2013). This switch can be mitigated by the anti-inflammatory cytokine IL-10 (El Kasmi and Stenmark, 2015), suggesting multiple ways that MSCs modulate macrophage metabolism and thus phenotypic function. The reliance of M1 macrophages on glycolysis and the pentose phosphate pathway (PPP) appears to be related to two interruptions in the tricarboxylic acid (TCA)/Krebs cycle, which cause accumulation and exit of itaconate, succinate and citrate from the cycle. These metabolites are released

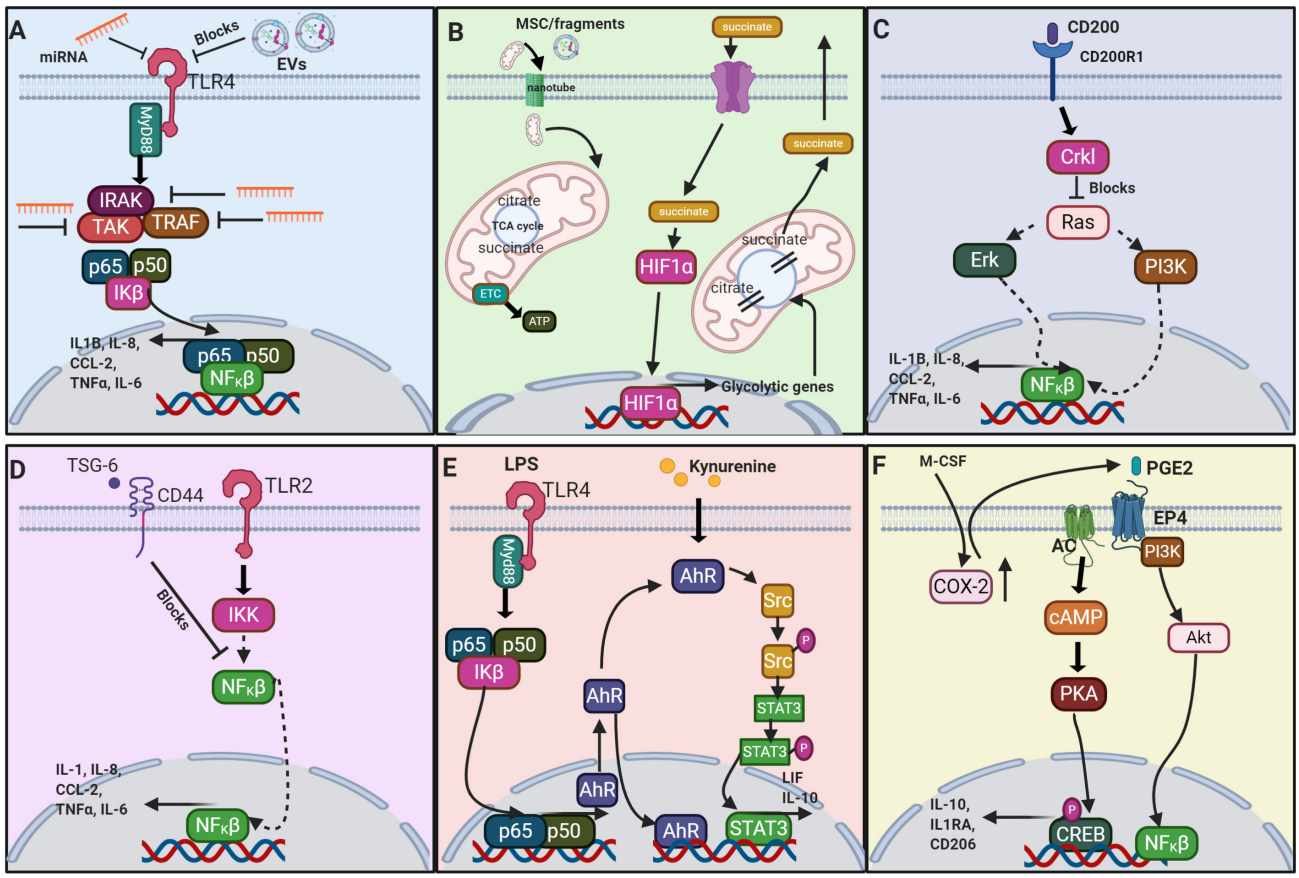

FIGURE 3 | Schematic of a selection of the proposed signaling pathways which result from MSC or MSC derived factors interacting with macrophages. **(A)** The signaling of extracellular vesicles (EV) via negative regulation of the TLR4 (toll-like receptor 4) (Abdi et al., 2018); **(B)** metabolite signaling via the TCA cycle (Viola et al., 2019); **(C)** CD200-CD200R1 interaction (Manich et al., 2019); **(D)** TSG-6 via negative regulation of TLR2 (Choi et al., 2011); **(E)** AhR via TLR4 signaling (Hinden et al., 2015; Zhu et al., 2018); and **(F)** PGE2 (Na et al., 2015; Xu et al., 2017). miRNA, microRNA; TLR4, toll-like receptor 4; EV, extracellular vesicle; MyD88, myeloid differentiation primary response 88; IRAK, interleukin-1 receptor-associated kinase; TAK, TGF-β activated kinase; TRAF, TNF receptor-associated factor; p65, NF-κβ p65 subunit; p50, NF-κβ p50 subunit; Iκβ, NFκβ inhibitor; NFκβ, nuclear factor kappa light chain enhancer of activated B cells; IL1β, interleukin-1 β; IL-8, interleukin-8; CCL-2, C-C motif chemokine ligand 2; TNFα, tumor necrosis factor α; IL-6, interleukin-6; MSC, mesenchymal stromal cell; HIF1α, hypoxia-inducible factor 1-α; TCA, tricarboxylic acid cycle; ETC, electron transport chain; ATP, adenosine triphosphate; CD200, cluster of differentiation 200; CD200R1, CD200 receptor 1; Crkl, Crk-like protein; Erk, extracellular signal regulated kinase; PI3K, phosphoinositide 3-kinase; TSG-6, TNFα-stimulated gene 6; CD44, cluster of differentiation 44; TLR2, toll-like receptor 2; IKK, inhibitor of nuclear factor NF-κβ kinase, LPS, lipopolysaccharide; AhR, aryl hydrocarbon receptor; Src, proto-oncogene c-Src; Src-P, phosphorylated Src; STAT3, signal transducer and activator of transcription 3; LIF, leukemia inhibitory factor; IL-10, interleukin-10; M-CSF, macrophage colony-stimulating factor; COX-2, cyclooxygenase 2; PGE2, prostaglandin E_2; EP4, E-type prostanoid receptor 4; AC, adenylate cyclase; Akt, protein kinase B; cAMP, cyclic adenosine monophosphate; PKA, protein kinase A; CREB, cAMP response element binding protein; IL-1RA, interleukin-1 receptor antagonist; CD206 (mannose receptor). Image created with BioRender.

from mitochondria, which both limits coupling of TCA to the electron transport chain (ETC) and also renders them capable of regulating cell metabolism. Succinate can stabilize HIF1α (**Figure 3B**) and thereby activate transcription of glycolytic genes, such that glycolysis is favored. Conversely, M2 macrophages appear to have an intact TCA cycle and so ROS are kept low and metabolites are not released to the cytoplasm (Viola et al., 2019). Upon receiving mitochondria from MSCs, M1-like (classical activation) macrophages polarized to M2-like (alternative activation) which resulted in a switch from glycolysis to oxidative phosphorylation. Therefore the exposure of MSCs to macrophages, and subsequent polarization to M2, is concomitant with a lower bioenergetic state with emphasis on catabolic pathways *in vitro* (El Kasmi and Stenmark, 2015; Vasandan et al., 2016). These catabolic pathways, involving β-oxidation of fatty acids, enhanced activity of 5′ AMP-activated kinase and reduced mTOR phosphorylation, are thought to rescue the macrophage from low tryptophan levels but have the obvious advantage of switching to energy conservation as well as a pro-regenerative TMφ state *in vitro* and *in vivo* (Phinney et al., 2015; Vasandan et al., 2016). During pathology, neural stem cells scavenge extracellular succinate, thwarting its utility by macrophages, in order to reduce infiltration of mononuclear phagocytes in neuroinflammation (Peruzzotti-Jametti et al., 2018). MSCs releasing insulin-like growth factor-2 under hypoxic conditions reprogram maturing macrophages to OXPHOS metabolism

to improve neuroinflammation in a mouse model of Multiple Sclerosis (Du et al., 2019). These studies allude to targeting the metabolic programs of macrophages to regulate or restore the homeostatic balance of the milieu as potential therapeutic approaches.

Macrophages are in large part responsible for the development of atherosclerotic plaques since their activation by IFNγ produces foam cells, which will proceed to form unstable lesions in the intima of arteries. The elevated expression of scavenger receptors and CD36 on foam cells allows for increased uptake of low density lipoprotein (LDL) (Murray and Wynn, 2011) and release of cytokines locally that influence atherosclerosis pathology *in vivo* (Tedgui and Mallat, 2006). Other macrophage phenotypes have been identified in the plaque and can be atheroprotective (i.e., Mhem) (Boyle et al., 2009), pro-atherogenic (i.e., Mox) (Kadl et al., 2010) or both (i.e., M4) (Erbel et al., 2015). In an atherosclerotic mouse model, the use of skin-derived or human amnion-derived MSCs decreased plaque size in the arteries *in vivo* (Li et al., 2015; Wei et al., 2019). MSCs are implicated in reducing the aggregation of TMφ in the arterial intima (Shoji et al., 2011), inhibiting the formation of foam cells by elevating the number and function of Tregs *in vivo* (Wang et al., 2015) and by decreasing TNFα release (Li et al., 2015). All of these steps require regulation of TMφ polarization and modulation of the phenotypes in the plaque (Yang et al., 2020). In this way, MSCs sense the inflammatory environment and attempt to mitigate TMφ inflammatory responses.

Oxidative Stress

MSCs counter oxidative insult by expressing antioxidant enzymes and heat shock proteins and upregulating redox-sensitive factors, such that lipid peroxidation and hydrogen peroxide (H_2O_2) and superoxide ($O_2^{\cdot-}$) radical species are decreased *in vitro* (Oh et al., 2014). If free radical quenching, antioxidant production, switching of TMφ bioenergetics and mitochondrial transfer is all viewed as management of oxidative stress then this constitutes a significant feature of the interaction of MSCs with TMφ at sites of inflammation. Stanniocalcin-1, secreted by MSCs, decreases reactive oxygen species (ROS) generation, including mitochondrial ROS and suppresses the activation of the nucleotide-binding domain and leucine-rich repeat pyrin 3 (NLRP3) inflammasome *in vitro* (Oh et al., 2014). The NLRP3 inflammasome in activated macrophages senses damage-associated molecular patterns (DAMPS) and generates IL-1β to initiate the inflammatory cascade. Its activity can be quenched by co-culture with umbilical cord blood MSCs (Shin et al., 2016). Furthermore, when these MSCs were incubated with recombinant human IL-1β, their COX-2 expression was upregulated, and this suggests the idea of a feedback loop between the IL-1β from the inflammasome and MSC immunosuppression. In this case direct COX-2/PGE2 signaling is responsible for immunosuppressive effects on the inflammasome, in the absence of NO and IDO effects (Shin et al., 2016).

Reactive oxygen species also play a role in macrophage polarization in the heart. Resident cardiac TMφ are thought to be lost with age or after myocardial infarction (MI) and replacement may be inferior, due to lack of resident cells to engage in proliferation or the pro-inflammatory activity of monocytes recruited from the bone marrow (Weinberger and Schulz, 2015). In an MI study in mice, less apoptotic cardiomyocytes were observed in the infarct zone, after MSC infusion, and although both M2 and M1 macrophage levels were decreased, M2 was proportionately increased in the heart but not bone marrow *in vivo* (Dayan et al., 2011). Rat and mouse models of MI, with MSC administration, observed increases in alternative activation (M2-like) TMφ at the transplant site *in vivo* (Ben-Mordechai et al., 2013; Ishikane et al., 2013). $CD146^+$ MSCs performed better than MSCs alone in a model of myocardial regeneration and this was attributed to a reduction in reactive oxygen species by the expression of CD146, an integral perivascular marker (Zhang et al., 2019a). In a follow up study, the total number of macrophages in the hearts of the mice did not vary after MSC transplantation but the ratio of M2:M1 increased (Zhang et al., 2019b). Injection of TNFα alongside the MSC transplantation abrogated the reparative effects of the MSCs *in vivo*. Although much is still unknown about macrophage subsets in the heart, MSCs appear to drive regeneration by reprogramming TMφ phenotypes.

Message in a Bottle – MSC-Derived Extracellular Vesicles

The paracrine-mediated immunomodulatory factors secreted by MSCs are not solely attributed to the release of soluble molecules that act directly on cells in the local environment but also by uptake of those packaged in extracellular vesicles (EVs). MSC-derived EVs (MSC-EVs) are implicated as a cell-free product with, in some cases, comparable effects to infused MSCs, thus rendering MSC-EVs attractive candidates as therapy. Cultured MSCs secrete EVs that can be collected and subsequently isolated from the conditioned media (CM), enabling studies to perform comparisons using MSC-CM and MSC-EVs to determine the direct influences of MSC-EVs. Together, the ability to harness the therapeutic effects of MSCs without the need to deliver the cells make MSC-EVs attractive treatment alternatives under growing investigations in pre-clinical and clinical trials (Harrell et al., 2019a).

Apoptotic bodies (>1,000 nm in diameter) are the largest of the EVs which bud from MSCs during apoptosis. Microvesicles (MVs) are typically 100-1,000 nm in diameter and bud from the plasma membrane, and exosomes are the smallest EVs with diameters measuring 30–200 nm and result from budding of the late endosome membranes (Harrell et al., 2019b). The cargo packaged into MSC-EVs include many of the soluble cytokines and molecules discussed herein as well as other proteins, enzymes, organelles, lipids, metabolites, nucleic acids, and non-coding RNAs, all of which are comprehensively discussed in the context of inflammatory disease by Harrell et al. (2019b). The cargo that relays the "messages" to distant sites is still elusive yet is suggested to be highly specific (Baek et al., 2019).

MSC-derived exosomes are capable of inducing macrophage polarization to the alternative activation phenotype by several proposed mechanisms. MSC-derived exosomes have been attributed to macrophage polarization to the alternative

activation phenotype with increased production and secretion of AREG by the described PGE2-dependent mechanism *in vitro* (Ko et al., 2020), suppression of the infiltration of classical activation macrophages and associated pro-inflammatory signaling (Mao et al., 2017; Willis et al., 2018a), and improved histoarchitecture by TMφ remodeling *in vivo* (Willis et al., 2018a) demonstrating alternative immunomodulatory delivery modalities that too can be supplied by MSCs (**Figure 3A**). Although the MSC-derived exosome-mediated effects are the result of the comprehensive mediators contained, depicting specific microRNA (miRNA) (Essandoh et al., 2016), cytokines, metabolites, and other molecules (Willis et al., 2018b) of which the EVs are comprised, may serve to identify other targeted therapeutics.

MSC-derived exosomes have been widely investigated in a number of pre-clinical studies of inflammatory diseases. TMφ induced to a classical activation phenotype and pro-inflammatory function that perpetuates inflammatory signaling were altered by MSC-derived exosomes to the alternative activation phenotype, resulting in attenuation of pathological severity in lung injury (Morrison et al., 2017; Wang et al., 2020), colitis (Mao et al., 2017), cardiomyopathy (Sun et al., 2018), retinal damage (Yu et al., 2016a), musculoskeletal conditions (Zhang et al., 2016; Cosenza et al., 2017), chronic wounds (Lo Sicco et al., 2017) and spinal cord injury (Lankford et al., 2018). MSC-derived exosomes not only mediated cellular improvements through TMφ, but also promoted the survival (Sun et al., 2018) and cytoprotection (Cosenza et al., 2017) of other vital tissue-specific cells *in vivo*. The mechanisms of action for MSC-derived exosomes remain to be fully elucidated, as multiple mediators are involved, and thus presumably more than one mechanistic effect, exerting comprehensive benefits during pathology.

Organelle Donation and Bioenergetics

Organelles such as mitochondria too can be shuttled from MSCs to TMφ to support higher demands of macrophage physiological functions. For example, stimulation of TMφ phagocytosis in acute respiratory distress syndrome (ARDS) was elicited via nanotube transfer of MSC mitochondria to macrophages *in vivo* (Jackson et al., 2016). In addition, MSCs under intracellular oxidative stress *in vitro* will shuttle damaged mitochondria (containing an excess of oxidized and nitrosylated proteins) into microvesicles for extrusion to improve their bioenergetics *in vitro*. Simultaneous de-sensitization via miRNA-containing exosomes from the MSCs mitigates the activation of TMφ, which permits phagocytosis and re-use of the donated mitochondria by TMφ, a mechanism that promotes the survival of MSCs by outsourcing mitophagy (Phinney et al., 2015). Macrophages co-cultured with MSCs have shown enhanced phagocytic activity and this may be orchestrated by nanotube/EV-mediated mitochondrial transfer from MSCs (Ibrahim et al., 2014; Phinney et al., 2015; Ko et al., 2020). Although more evidence is necessary to determine the advantage of using MSC-EVs over MSCs, alternative strategies to elicit the comprehensive effects from MSC-macrophage crosstalk are promising.

CONTACT-DEPENDENT COMMUNICATION

The complexities of the intercommunication between MSCs and TMφ may never be completely teased apart, and therefore we should expect possibilities of both indirect and direct mechanisms working in tandem to promote improvements to a pathological milieu. Moreover, the spatiotemporal microenvironment will continue to be altered to resolve inflammation and restore homeostasis, necessitating multiple functions of both the macrophages and MSCs. The dynamic heterogeneity of functions of both cell types are no coincidence; the integral crosstalk is the forefront to not only resolving pathology but improving our understanding of prophylactic measures to prevent disease. The observations of a number of *in vivo* investigations are presented in **Table 2**.

Phagocytosis of Dead/Apoptotic Cells

Mechanisms involved in recognition of apoptotic cells and the consequential removal by phagocytosis, involve receptors classed as C-type lectin, vitronectin (with assistance from CD36 and thrombospondin on apoptotic cells), phosphatidylserine, scavenger proteins (bind apoptotic, necrotic cells, opsonized pathogens and cell debris), TLRs and macrophage antigens. Phagocytosis of apoptotic cells has been shown to inhibit macrophage production of a range of cytokines, except TGFβ1, PGE2 and platelet activating factor (PAF) *in vitro* (Fadok et al., 1998). In general, the result of phagocytosis appears to be immunosuppression. Recently, a novel cell contact-dependent mechanism demonstrated a lipoprotein receptor protein mediated uptake of MSC-derived cytoplasmic components, or processing bodies, by monocytes and macrophages resulted in reprogramming to an anti-inflammatory function. Reprogrammed monocytes and macrophages were able to significantly suppress activated helper T cells proliferation *in vitro* and mitigate inflammation in a small animal model of LPS-induced lung inflammation (Min et al., 2020). In a pre-clinical model of asthma, polarization of alveolar TMφ was accompanied by phagocytosis of PKH26 + MSC and upregulation of TGFβ and IL-10 mRNA (Braza et al., 2016) and M2 markers *ex vivo* were only expressed on macrophages which had ingested MSCs.

Indeed the daily clearance of fetal apoptotic stromal cells by maternal resident lung macrophages, stimulating IL-10 release and IL-1β suppression, facilitates immunotolerance *in vivo* (Abumaree et al., 2006; Galipeau and Sensebe, 2018). Efferocytosis, a term originally coined in 2003, is the targeted removal of apoptotic cells, as well as cells dying through the many other forms of cell death (Henson, 2017). Phagocytosing macrophages clear apoptotic MSCs, and this process precipitates intracellular signaling in macrophages to downregulate TNFα and NO production, in favor of TGFβ1 and IL-10 *in vitro* (Braza et al., 2016; de Witte et al., 2018). Therefore, MSCs may take part in immunoregulation and macrophage polarization even after becoming apoptotic.

These studies allude to the state of MSCs when delivered *in vivo*. Freshly thawed cells, in general, have a higher metabolic

TABLE 2 | A selection of *in vivo* studies using therapeutic MSCs with described pathology, source of MSCs, delivery route and outcomes.

Pathology	MSC source	Delivery route	Results/mediators observed	Citation
Acute lung injury	mBM-MSC	Intratracheal	↓neutrophils, NOS2, ↑Ym1, Arg1	Ionescu et al., 2012
Allergan-induced inflammation	mBM-MSC	IV	↓total cell count, IL-4, IL-13,IL-17 in BAL, ↓eosinophils, neutrophils in airway, ↑MSC in lungs	Xu et al., 2015
Asthma	mBM-MSC	IV	↑MSC in airways, *ex vivo ↓IL-6, IL-1β, NOS2*	Cui et al., 2020
Atherosclerosis	mBM-MSC	IV	↑Tregs, TGFβ, IL-10	Wang et al., 2015
Cardiomyopathy	mMSC-Exos	IV	↓apoptosis cardiomyocytes ↓M1, IL-1, IL-6, TNFα	Sun et al., 2018
Colitis	hUC MSC-Exos	IV	↓TNFα, IL-1β, IL-6, ↑IL-10	Mao et al., 2017
Colitis	canine ADMSCs	IP	↑TSG-6, IL-10, M2 ↓TNFα, IL-6,	Song et al., 2018
Collagen-induced arthritis	mBM-MSC	IV	↓serum TNFα, IL-1β	Luz-Crawford et al., 2016
Corneal allotransplantation	hBM-MSC	Peritransplant IV	↓IL-6, IL-1β, IL-12, ↓actn APCs, ↑TSG-6	Oh et al., 2012
Corneal injury	mBM-MSC	IV	↓IL-1β, inflam cell infiltration	Amouzegar et al., 2017
Cutaneous wound	hUC-MSC-Exo	Injected into wound	↓inflam cell infiltration, TLR4, p-P65, M1	Ti et al., 2015
Dermal injury	mBM-MSC	IV	↑transdifferentiation to skin cells	Sasaki et al., 2008
Dust mite asthma	mBM-MSC	IV	↑M2, IL-10, TGFβ1, ↓IL-6, MSCs only in M2s.	Braza et al., 2016
E. coli pneumonia	hBM-MSC	IV/intranasal	↓MIP-1α, MIP-1β, IL-27, IL-6, TNFα, ↑phagocytosis	Jackson et al., 2016
Experimental autoimmune encephalomyelitis	hUCMSC	IV	↓IL-1β, IFNγ, IL-17, ↑PD-L1, IGF-2	Du et al., 2019
GvHD	hBM-MSC	IP	↓GvHD effector T cells	Galleu et al., 2017
Intracerebral hemorrhage	rBM-MSC	IV	↓apoptosis, TMΦ, neutrophils, iNOS, MMP-9	Chen et al., 2015
Ischemia-reperfusion injury	Cardiac MSC	Intracardiac injection	↑CCR2+, CXCR1 + TMΦ,	Vagnozzi et al., 2020
Kidney ischemia-reperfusion	mBM-MSC	IV	↑MCP-1, MIP1α, IL-1β, 1L-10, TGFβ	Luk et al., 2016
LPS-induced abortion	mBM-MSC	IP	↓TNFα, IFNγ, IL-1β, IL-27, IL-6, ↑TSG-6	Li et al., 2019
Lung injury	mBM-MSC	IV	↓TNFα, IL1-RA	Ortiz et al., 2007
Lung injury	hBM-MSC	IV	↓neutrophils, ↑IL10, KGF	Devaney et al., 2015
Lung injury	hADMSC-Exo	IV, intratracheal	↓neutrophils in BAL, NFκβ, ↑IL-10, Arg1, miR-27a-3p in alveolar TMΦ	Wang et al., 2020
Myocardial infarction	hBM-MSC/hUC-MSC	IV	↓IL-1β, IL-6, apop cardiomyocytes, ↑IL-10, CD206+	Dayan et al., 2011
Osteoarthritis	hBM-MSC-CM	Intra-articular	↓MMP-13/TIMP-1, ↑autophagy chondrocytes	Chen et al., 2019
Retinal inflammation	hBM-MSC	IV	↓CD3+ cells ↑Treg	Ko et al., 2020
Retinal injury	mADMSC-Exos	Intravitreal	↓apoptosis, MCP-1, MΨ infiltration	Yu et al., 2016a
Sepsis	mBM-MSC	IV	↓TNFα, IL-6, ↑IL-10	Nemeth et al., 2009
Sepsis	Apoptotic rADMSC	IP	↓TNFα, MMP-9, NFκβ	Chang et al., 2012
Spinal cord injury	rMSC-Exos	IV	Exos in CD206 + Mψ only	Lankford et al., 2018
Spinal cord injury	hBM-MSC	Injected into injury	↑IL-4, IL-13, M2 TMΦ, ↓TNFα, IL-6, M1 TMΦ	Nakajima et al., 2012
Spinal cord injury	Dental pulp MSC-CM	Injected into injury	↑TGFβ, VEGF, CD206+, MCP-1, ED-Siglec 9,	Matsubara et al., 2015
Traumatic brain injury	hBM-MSC	IV	↑TIMP-3, ↓VEGF-A, blood brain barrier permeability	Menge et al., 2012

activity, greater percentage of apoptotic cells and a higher necrotic fraction than culture rescued cells (Antebi et al., 2019). MSCs used within 24 h of thawing, show compromised T cell suppression, increased susceptibility to lysis by complement or immune cells and shortened persistence *in vivo* with intravenous (IV) administration (Moll et al., 2016). It has also been shown that roughly 50% of IV transfused mouse MSCs become trapped in the lung and are ultimately phagocytosed by lung resident macrophages (Nemeth et al., 2009). In an ischemia-reperfusion injury model in mice, cardiac MSC injection improved heart function, not by production of new cardiomyocytes but by induction of $CCR2^+$ and $CX3CR1^+$ TMφ. Changes in the local extracellular matrix content of the peri-infarct border zone *in vivo* occurred whether the MSCs were live or freeze-thawed (non-viable) and could be substituted by a chemical inducer of the innate immune response (zymosan) (Vagnozzi et al., 2020). Therefore, this may be an indirect effect of the MSCs and not paracrine-mediated, but nevertheless, MSCs whether viable, intact or even apoptotic, cell signaling still produces a beneficial effect on the resulting macrophage functional output.

It is therefore possible that MSCs can be apoptotic, metabolically inactivated or fragmented even (membrane particles) and still exert immunomodulation (Luk et al., 2016; Goncalves et al., 2017). Apoptotic adipose-derived MSCs were able to improve survival of rats in a model of sepsis by decreasing TNFα levels in circulations as well as the frequencies of systemic and splenic helper and cytotoxic T cells (Chang et al., 2012). Evidence supports that apoptotic cells were more potent than viable cells in lung, kidney injury and ischemia-reperfusion models (Sung et al., 2013). Furthermore, the deliberate perforin-mediated induction of apoptosis of MSCs by cytotoxic cells in a murine model of GvHD, appeared necessary for the success of the MSC infusion (Galleu et al., 2017). Induction of caspase 8 and apoptosis was necessary for immunosuppression *in vivo* and patients with high cytoxicity were more likely to respond to MSCs, indicating a bifurcation of patient responses. Intraperitoneally (IP)-delivered MSCs were sequestered in the phagocytes in lymph nodes and IV-delivered MSCs homed to the lungs. These apoptotic MSCs only improved GvHD outcomes when delivered IP, and not IV, and no IDO was induced by IV-administered MSCs. Regulation of pro-inflammatory Th1 and Th17 cells by MSC-derived PGE2 only occurred in the presence of $CD14^+$ cells in PBMC cultures, therefore indicating a reliance on myeloid cells for immunoregulatory mechanisms (Rozenberg et al., 2016).

The heat inactivation (HI) protocol of Luk et al. (2016) introduced in 2016 incubated MSCs for 30 min at 50°C (HI MSCs) and this resulted in the lack of a secretory profile, no proliferative or metabolic activity and a disintegration of the cell without heat shock protein release. HI MSCs did not inhibit T cell proliferation but were able to reduce TNFα release by monocytes challenged with LPS *in vitro* (Luk et al., 2016). This points to a non-specific immunosuppression, i.e., independent of cell viability, by the reticuloendothelial system of the host (Poon et al., 2014). Indeed, studies in our laboratory, using MSC suppression of TNFα release by THP-1 macrophages, have found both freshly thawed and cultured MSCs able to exert immunosuppressive effects and yet the culture-rescued cells generally have few apoptotic and necrotic cells post harvest, as mentioned earlier (Pradhan P et al. BioRxiv doi: https://doi.org/10.1101/2020.09.12.294850).

Aryl Hydrocarbon Receptor on MSCs

Aside from the secretome and engulfment of MSCs, a proposed mechanism mediated by MSCs resulting in macrophage polarization, or macrophage phenotype "plasticity," is by the activation of the aryl hydrocarbon receptor (AhR). This MSC receptor responds to environmental stimuli and contributes to both physiological cell development and immune regulation (Abney and Galipeau, 2020). The AhR, when bound by ligands of environmental pollutants, translocates from the cytoplasm to the nucleus and facilitates AhR-related transcription of genes, which typically elicit immunotoxicological effects. For example, MSCs upregulate cytochrome P450 isoforms, *cyp1a1* and *cyp1b2* genes in response to cockroach allergen *in vitro* (Xu et al., 2015). This AhR receptor activation is kynurenine-mediated and results in immunosuppressive alterations [decreased IL-6 expression and enhanced leukemia inhibitory factor (LIF) *ex vivo* (Hinden et al., 2015)] (**Figure 3E**). This suggests an immunomodulatory potential of MSC directly regulated by AhR.

In mice treated with MSCs prior to intratracheal cockroach extract (CRE) challenge, there was a significant decrease in bronchial inflammation and goblet cell hyperplasia. Isolated lung TMφ showed a significant increase in alternative activation (M2-like) marker expressions (e.g., Arg1, FIZZ1, and Ym1) relative to CRE treatment alone, suggesting treatment with MSCs polarized macrophages to an alternative activation phenotype under allergen-induced pulmonary inflammation *in vivo* (Cui et al., 2020). The IDO inhibitor 1-methyl tryptophan can activate AhR in MSCs *in vitro* (Lewis et al., 2017) and the AhR-Src-STAT3-IL10 signaling pathway has been pivotal to controlling inflammatory macrophages *in vitro* (Zhu et al., 2018). The correlation between this signaling pathway and immunomodulatory mechanisms by MSCs may be a key axis for targeted approaches.

CD200, TSG-6, and Hormone Receptors

Another contact-dependent interaction implicated in macrophage polarization is the CD200/CD200R1 receptor complex. CD200 (OX-2) is a transmembrane glycoprotein and its counterpart, CD200-R1, is found on myeloid cells and T cells. Interestingly, the role of CD200 cannot be fully extricated from soluble tumor necrosis factor stimulated gene-6 (TSG-6) signaling. In an LPS-induced abortion mouse model, TSG-6-silenced or CD200-silenced MSCs exhibited a higher embryo resorption rate and both had higher levels of TNFα, IFNγ, and induced nitric oxide synthase (iNOS) in the decidua than non-silenced control MSCs supporting the CD200- (cell-mediated) and TSG-6-dependent (i.e., paracrine-mediated) mechanism (Li et al., 2019) (**Figure 3C**). This evidence gives credence to the idea that MSCs exert immune tolerance by both cell-contact as well as paracrine-mediated mechanisms.

The ability of bone marrow MSCs to suppress TNFα release by IFNγ-primed THP-1 macrophages appeared correlated to the levels of CD200 (Pietila et al., 2012). High expression of CD200 on umbilical cord derived MSCs was associated with improved immunomodulatory effects *in vitro* and lack of CD200 expression was correlated to poor suppressive capacity of the MSCs, suggesting a link between CD200 expression on MSCs and suppression of pro-inflammatory macrophage signaling (Pietila et al., 2012). TSG-6 released by MSCs is a signaling molecule that has been the focus of several pathological conditions. TSG-6 deletion in MSCs abrogated the ability to repair corneal damage, myocardial infarct and aid in corneal allograft survival (Oh et al., 2012). The role of TSG-6 signaling has been explored in inflammatory bowel disease (IBD), where canine adipose tissue-derived MSCs induced polarization of TMφ in murine IBD, resulting in more M2 TMφ released into the colon and improvements in disease activity index (Song et al., 2018). TSG-6, released by the MSCs, prevented blood brain barrier (BBB) disruption in intracerebral hemorrhage in rats and reduced the density of microglia/macrophages at the hemorrhage site (Chen et al., 2015). TSG-6 has a known interaction with the CD44 receptor on TMφ, which blocks TLR2-mediated translocation of nuclear factor kappa κβ (NFκβ) to the nucleus alleviating inflammatory signaling *in vitro* (Choi et al., 2011).

In pathological conditions, MSCs have been shown to block the translocation of NFκβ to the nucleus, indicative of the TSG6/TLR2/NFκβ pathway. LPS and IFNγ trigger intracellular signaling pathways, via degradation of Iκβ, which frees NFκβ to translocate to the nucleus to bind promoters of pro-inflammatory mediators (**Figure 3D**). LPS-induced lung injury was lessened by treatment with MSCs or MSC-CM, with alveolar macrophages showing heightened Ym1 and decreased iNOS (NOS2) compared to untreated controls *ex vivo* (Ionescu et al., 2012). However, the signaling pathways of activated resident macrophages are complex and difficult to study *in vivo*, with considerable heterogeneity of response to different stimuli. The transcriptome of activated macrophages revealed nine distinct activation programs, a spectrum of activation much more advanced than the M1/M2 classification conventions (Xue et al., 2014).

Apart from modulation of TMφ phenotypes, MSCs also play a role in blocking the differentiation of steady state myeloid progenitors under inflammatory conditions and the subsequent infiltration of inflammatory effector cells at the site of inflammation. Under homeostatic conditions, bone marrow-derived MSCs support hematopoiesis, maintaining hematopoietic stem cells (HSCs) in an undifferentiated state via trophic factor release. Under inflammatory conditions (i.e., high levels of IFNγ, IL1β, and TNFα), however, HSCs undergo myelopoiesis, resulting in their differentiation to macrophages and neutrophils. CD200 expressed on MSCs is proposed to be responsible for the suppression of inflammation and the maintenance of myeloid progenitors in an undifferentiated state *in vitro* and *in vivo* (Amouzegar et al., 2017). In a mouse model of corneal injury, systemically administered control MSCs showed a fivefold induction of myeloid progenitors in the cornea and a concomitant reduction in inflammatory cells and IL-1β, compared to mice injected with (silenced) CD200-shRNA-treated MSCs (Amouzegar et al., 2017).

Besides PGE2-EP4, TSG-6-CD44, and CD200-CD200R1, signaling between progesterone receptors (PR) and glucocorticoid receptors (GR) on microglia, the macrophages of the CNS, has been implicated in the triggering of microglial polarization *in vivo* (Xu et al., 2017). Progesterone has been shown to be neuroprotective in pre-clinical models of traumatic brain injury, by inhibition of microglial activation and prevention of inflammatory cytokine release (Lopez-Rodriguez et al., 2015). Inhibition of PR and GR by mifepristone partly blocked human placental MSC-driven polarization of macrophages. In this study, the basal release of soluble factors by MSCs suggested TGFβ as a key mediator of the resulting immunomodulation (Abumaree et al., 2013).

MSC-macrophage interactions in bone (re)modeling appear to be paracrine- and contact-mediated via CD200/CD200R *in vitro* (Varin et al., 2013). Osteoclasts (bone resorbing cells) can differentiate from hematopoietic precursor cells or other macrophage lineage cells. Activated TMφ, which release pro-inflammatory cytokines, can disrupt the balance of osteoclast-mediated bone resorption and osteoblast-mediated bone formation, resulting in bone loss (Yang and Yang, 2019). However, depletion of TMφ during intramembranous bone deposition in fracture repair led to impaired healing (Alexander et al., 2011). Soluble CD200 can inhibit differentiation of osteoclast precursors and inhibit receptor activator of nuclear factor kappa-β ligand (RANKL) signaling. MSCs expressing CD200 can block osteoclast formation and resorption pit activity *in vitro* (Varin et al., 2013) and CD200R inhibition can result in hyperactivation of macrophages and increased susceptibility to autoimmune diseases (Wright et al., 2003). Future research should unravel the reliance on contact dependent vs. soluble mediators for bone regulation and pathology.

MSCs ALTERED BY MACROPHAGES

Macrophage conditioned media, as well as co-culture with MSCs, can influence MSCs viability and secretome (Freytes et al., 2013). M2 macrophages are reported to produce more osteoactivin/gpnmb and thereby activate the ERK/JNK signaling pathway to assist MSC survival, proliferation and migration (Yu et al., 2016b; Xu et al., 2017). LPS-induced TNFα release by macrophages can stimulate MSCs to secrete growth factors that promote tissue repair (Crisostomo et al., 2008) and drive MSCs to release inflammatory cytokines (Abumaree et al., 2013).

It is notable that M1 macrophage-MSC co-cultures demonstrated markedly higher upregulated genes compared to than equivalent M2 macrophage-MSC co-cultures (Espagnolle et al., 2017), verifying that the macrophage program can specify gene expression and cell-mediated immune responses. Upregulated genes in M1-MSCs cultures included *IDO*,

COX2 (immunosuppressive genes), *PDL-1*, *CD54* (MSC and T lymphocytes), *CXCL9* and CXCL10 (involved in T cell trafficking). M1-primed MSCs showed stronger inhibition of T cell proliferation, likely through a homotypic CD54 synapse between M1 macrophage and MSC (Espagnolle et al., 2017).

Other evidence of the effects of macrophage-primed MSCs can be found in orthopedic research, and relate to the multi-tissue compartment of the joint. M2 macrophages, co-cultured with MSCs, drive the expression of alkaline phosphatase, osteogenic markers and bone mineralization to regenerate bone (Champagne et al., 2002) and the expression of chondrogenic and clonogenic genes, to aid cartilage formation (Sesia et al., 2015). Similarly, exosomes isolated from LPS-treated monocytes increased gene expression of Runx2 and BMP-2 in human MSCs upon exposure *in vitro* (Ekstrom et al., 2013). Synovial M1 macrophages promote upregulation of proteolytic enzymes in osteoarthritis and negatively impact MSC chondrogenic effects on chondroprogenitors (Fahy et al., 2014). These key investigations shed light on the potential MOAs of MSCs in musculoskeletal indications, however, the dark side of the molecular crosstalk between MSCs and TMφ reveals how these potential MOAs can be exploited during carcinogenesis.

Although this review is intended to focus on MSCs administered as therapy, several studies have also reported a central crosstalk between MSCs and TMφ in the context of cancer. Given that MSCs are defined as a cultured cell type and their *in vivo* identity prior to isolation still remains unclear [i.e., pericyte-like (Caplan, 2008)], explorations to reveal the influences of MSCs within the tumor microenvironment are limited. Often, culture expanded MSCs are co-cultured with cancer cells and TAMs isolated from tumors or injected directly into the *in vivo* tumor microenvironment to investigate the influences of MSCs. Furthermore, it is challenging to elucidate the phenotypes, functions, and crosstalk attributed to the various cells within the *in vivo* tumor microenvironment that may, collectively, promote or mitigate cancer progression.

Like TMφ, the paradigm of polarized responses resulting from TLR signaling has also been described for MSCs (i.e., MSC1 and MSC2), suggesting pro- or anti-inflammatory effects (Tomchuck et al., 2008; Waterman et al., 2010). Waterman et al. (2012) suggested that MSC1 attenuated tumor growth *in vitro* and *in vivo*, whereas MSC2 had the opposite effect of promoting tumor growth and metastasis, linking the secretory profiles of MSCs directly to alterations to cancer cells. The changing tumor microenvironment likely alters, "educates," or even "hijacks" (Quail and Joyce, 2013) MSCs as well as tumor-associated macrophages (TAMs). For example, key findings have linked inflammation and cancer progression by elucidating the roles of polarized TAMs and their activation of MSCs. Anti-tumor effects have been attributed to M1-like TAMs and, in contrast, multiple aspects of tumor progression are correlated with the suppressive program of M2-like TAMs. Inflammation in the tumor microenvironment produced an M1 phenotype of TAMS which, in turn, induced an immunosuppressive profile of MSCs, expressing high levels of iNOS and MCP1. Further recruitment of TAMs mediated by MCP1 secreted from MSCs along with IL-6 led to polarization into an M2-like phenotype which promoted tumor growth (Jia et al., 2016). Our contextual understanding regarding the crosstalk between TAMs and endogenous cells during carcinogenesis is far from being fully understood. A greater appreciation of the crosstalk between MSCs and TAMs as well as the development of cancer stem cells in cancer research can be found in more focused reviews by Papaccio et al. (2017) and Ridge et al. (2017).

CONCLUSION

Scientific understanding is continually enriched and reshaped by technological advancements, research methodologies and new discoveries. As our understanding of monocytes and macrophages has evolved recently, so has our viewpoint about MSCs. There is now broad agreement that MSCs are not in fact stem cells and likely do not exhibit multipotency when delivered *in vivo*; rather, they are potent signaling cells with great plasticity, that interact dynamically with their microenvironment, e.g., with TMφ, to modulate and control immune homeostasis and produce, or help produce, various pro-regenerative signals.

As with current assays that evaluate *in vitro* functional or therapeutic potencies of MSCs, a developed assay based on an identified MOA needs to account for many considerations that may obscure the reproducibility of results – highlighting the importance of standardization of all processes from harvest to delivery of MSCs. One must consider the biological variation of each donor and alterations imparted by different manufacturing processes including, how the cells were isolated, stored, shipped, cultured, expanded, and delivered (e.g., route, timing, and dose). A major realization is that MSCs are mainly administered by intravenous infusion, destined for entrapment in the lungs – a tissue that may be far from the site of pathology. Thus, the applicability of a given MOA with respect to the route of delivery and site of pathology for treatment must be considered, although the predominant therapeutic effects of MSCs could be via paracrine activities (Giri and Galipeau, 2020). Furthermore, we refer to therapeutic MSCs as a culture-based cell type confined by an identity characterized *ex vivo* that are then re-introduced to an *in vivo* milieu that is highly variable from patient to patient. This alludes to the difficulty in developing *in vitro* assays that are predictive of *in vivo* outcomes. The complexity of identifying and validating potential MOAs mediated by therapeutic MSCs bolster the need for deep and broad characterization of the cells especially using multi-omic analyses, better understanding of the critical process parameters that can help produce cells with consistent and reproducible quality, identification of the critical quality attributes that are predictive of the product quality and patient outcomes, standardization of processes and analytical methods, pertinent *in vitro* potency and safety assays, appropriate animal models for *in vivo* pre-clinical validation, and well-designed randomized controlled trials to evaluate clinical efficacy.

The evidences from pre-clinical studies, to date, suggest that MSC-macrophage crosstalk may play a critical role in their *in vivo* function and can be a potential MOA. These interactions are largely a result of the MSC secretome, including soluble factors,

mitochondrial donation, mediating complex pathological milieus characterized by pro-inflammatory, metabolic, proliferative, differentiative, hypoxic, REDOX mediators, along with some cell contact-dependent mechanisms. Together, these studies shed light on the various modes in which MSCs alter macrophage phenotype and, in so doing, can modulate local and systemic immunopathology to promote repair and restore homeostasis.

As mentioned earlier, the majority of mechanisms by which macrophages and MSCs interact have been discovered *in vitro* and much more *in vivo* studies are needed to tie in these *ex vivo* observations to those occurring in the body upon administration. Through correlation of *in vitro* functional assays to qualitative and quantitative *in vivo* effects, we should be able to identify potency assays which are more representative of *in vivo* performance and employ these to inform the manufacturing of MSCs for mainstream clinical therapy. The potential for MSC therapeutics lies in the ability to improve our understanding of how we can best harness their key communication mechanisms with other cells, and reproducibly promote the beneficial effects, ultimately translating benchtop discoveries to bedside MOAs to advance these promising therapies into clinic and the industry.

AUTHOR CONTRIBUTIONS

HS and AB contributed to the preparation, writing, and review of the manuscript. CY and KR contributed to the writing and review of the manuscript. All authors approved the submitted version.

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REVIEW
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The Role of Notch and Wnt Signaling in MSC Communication in Normal and Leukemic Bone Marrow Niche

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Notch and Wnt signaling are highly conserved intercellular communication pathways involved in developmental processes, such as hematopoiesis. Even though data from literature support a role for these two pathways in both physiological hematopoiesis and leukemia, there are still many controversies concerning the nature of their contribution. Early studies, strengthened by findings from T-cell acute lymphoblastic leukemia (T-ALL), have focused their investigation on the mutations in genes encoding for components of the pathways, with limited results except for B-cell chronic lymphocytic leukemia (CLL); in because in other leukemia the two pathways could be hyper-expressed without genetic abnormalities. As normal and malignant hematopoiesis require close and complex interactions between hematopoietic cells and specialized bone marrow (BM) niche cells, recent studies have focused on the role of Notch and Wnt signaling in the context of normal crosstalk between hematopoietic/leukemia cells and stromal components. Amongst the latter, mesenchymal stromal/stem cells (MSCs) play a pivotal role as multipotent non-hematopoietic cells capable of giving rise to most of the BM niche stromal cells, including fibroblasts, adipocytes, and osteocytes. Indeed, MSCs express and secrete a broad pattern of bioactive molecules, including Notch and Wnt molecules, that support all the phases of the hematopoiesis, including self-renewal, proliferation and differentiation. Herein, we provide an overview on recent advances on the contribution of MSC-derived Notch and Wnt signaling to hematopoiesis and leukemia development.

Keywords: Mesenchymal stromal cells, Notch, Wnt, leukemia, bone marrow niche

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INTRODUCTION

Bone marrow microenvironment (BMME) supports normal and clonal hematopoiesis, but also affects leukemia initiation, progression, and chemoresistance. Hematopoietic stem cells (HSCs) reside in a specialized BMME, where HSCs are tightly regulated (Cordeiro-Spinetti et al., 2015), functionally subdivided in two main compartments, i.e., the vascular niche that is close to the marrow vasculature, and the endosteal niche that is close to endosteum; however, the specific nature and functions of each niche still remain unclear (Morrison and Scadden, 2014;

Calvi, 2020). Within BM niches, HSCs interact with cellular components, including endothelial cells (ECs), mesenchymal stromal cells (MSCs), megakaryocytes (MKs), osteoblasts (OBs), specialized macrophages, and nerve fibers (Calvi et al., 2003; Wilson et al., 2007). The redundant and complex activity shared by these cellular components has made difficult the assessment of the precise role of each cell type. However, these cells are dynamically involved in the regulation of hematopoiesis, through soluble or membrane-bound molecules (receptors and ligands) (Morrison and Scadden, 2014). MSCs include adult stem cells with multilineage differentiation capacity, that give rise to many other stromal cell types, including osteoblasts, adipocytes, chondrocytes, and endothelial cells (Dominici et al., 2006). As observed, both *in vitro* and in animal models, MSCs are capable of reconstituting a functional hematopoietic microenvironment, expressing/producing cytokines, and growth factors necessary for the regulation of hematopoiesis (Muguruma et al., 2006; Pontikoglou et al., 2011). Consequently, MSCs are largely used in 2D and 3D *in vitro* or *ex vivo* co-culture systems as a surrogate of the BMME, thus representing a suitable model for evaluating the role of BMME on HSCs and leukemic cells (Jakubikova et al., 2016). MSCs, by either producing cytokines and chemokines or entering in direct contact with leukemia cells, activate cell transduction signals that tightly regulate normal and malignant hematopoietic cell survival, thus driving the chemoresistance-promoting effect of the BMME (Jacamo et al., 2014). Our and other groups have demonstrated that Notch and Wnt signaling pathways represent a major crosstalk used by MSCs to interact with BMME (Kamdje et al., 2011, 2012; Zhang et al., 2013; Takam Kamga et al., 2016a). Indeed, these two pathways are well documented for their pivotal functions during normal and malignant hematopoiesis. Even though their deep role is well known in some leukemia subtypes, such as T-ALL, they can play opposite functions, being either oncogenic or tumor suppressor. However, all studies eventually unravel a conserved and supportive role for MSC-derived Notch and Wnt pathways in leukemia.

MSCs

Mesenchymal stem/stromal cells (MSCs) are multipotent non-hematopoietic cells with multilineage differentiation capacity. According to ISCT (International Society for Cellular and Gene Therapy, MSCs could be defined according to three criteria; (i) spindle shaped and plastic-adherent cells in standard tissue culture plates; (ii) expression of mesenchymal markers (CD105+, CD73+, CD90+) and lack of hematopoietic markers (CD45-, CD34, CD14- or CD11b-, CD79a or CD19, and HLA-DR), and (iii) *in vitro* multipotent capability of differentiating into osteocytes, adipocytes, and chondrocytes (Dominici et al., 2006). There are several sources of MSCs including BM, cord blood, adipose tissue, and others (Krampera et al., 2007; Di Trapani et al., 2013; Petrenko et al., 2020). MSCs have become widely studied over the past 30 years for their potential clinical application in tissue engineering and regenerative medicine for bone and cartilage reconstruction and wound healing. Actually, *in vitro* and *in vivo* data support the evidence that one of the most important biological properties of MSCs is the immunoregulatory effect toward innate and adaptive immune effectors cells, such as T-, B-, and NK-cells, monocytes and dendritic cells in different inflammatory conditions, such as graft-versus-host disease (GvHD) (Collo et al., 2020). Indeed, migration, secretion, tissue regeneration, and immune regulatory properties of MSCs are synergistic and frequently rely on common signaling pathways, such as bone morphogenetic proteins (BMP) (Kong et al., 2013), platelet-derived growth factor (PDGF), Wnt, and Notch, especially inside BMME. Leukemia cells can interfere with the modulation of these pathways to improve biological function of MSCs toward a pro-leukemia supportive effect (Wang et al., 2015, 2016).

NOTCH SIGNALING IN MSCs

Notch Signaling: Structure and Activation

Notch signaling is a master and evolutionary pathway conserved from flies to human (Ntziachristos et al., 2014). The term Notch is related to the notched wing phenotype observed in flies carrying notch gene haploinsufficiency, as Notch is involved in tissue patterning (Morgan, 1917). Mammal Notch system involved 4 receptors of Lin/Notch family (Notch 1, Notch 2, Notch 3, and Notch 4) and 5 ligands of the Delta/Serrate/lag-2 (DSL) [Delta-like ligands (DLL-1, 3-4), Jagged1 and Jagged2] (**Figure 1**; Gordon et al., 2008; Ables et al., 2011). Notch receptors are single-pass transmembrane receptors, containing three domains: an extracellular domain, a transmembrane domain and an intracellular domain, the latter known as Notch intracellular domain (NICD). The extracellular unit consists of an epidermal growth factor (EGF)-like repeat domain, which participates to the ligand binding. There are 36 EGF-repeats domains in Notch1 and Notch 2, and 34 and 29 repeats for Notch3 and Notch 4, respectively. EGF-like repeats are followed by a Lin12/Notch/repeats (LNR) structure acting as a negative regulatory region (NNR), by preventing the ligand-independent cleavage of the receptor. The NICD presents the RBP-J-associated molecule (RAM) domain, six ankyrin repeats (ANK), nuclear localization sequences (NLS), a transactivation domain (TAD) required for activating transcription, and a proline-, glutamate-, serine-, and threonine-rich (PEST) domain which regulates NOTCH degradation. Initially, Notch receptors are transcribed and translated as 210–300 kDa large precursor molecules. A series of post-translational modifications are required for the precursors to acquire their active form. The intact precursor molecules are first glycosylated in the endoplasmic reticulum (ER) by O-fucosyletransferase (Pofut-1 in mammals), which adds fucose to serine or threonine sites on specific EGF-like repeats. The glycosylated precursors are then cleaved in the trans-Golgi network into two subunits by furin-like convertases (S1-cleavage). This cleavage converts the precursor molecule into the non-covalently linked Notch extracellular domain (NECD) and transmembrane-Notch intracellular domain (TM–NICD) complex. This is then further glycosylated by enzymes of the

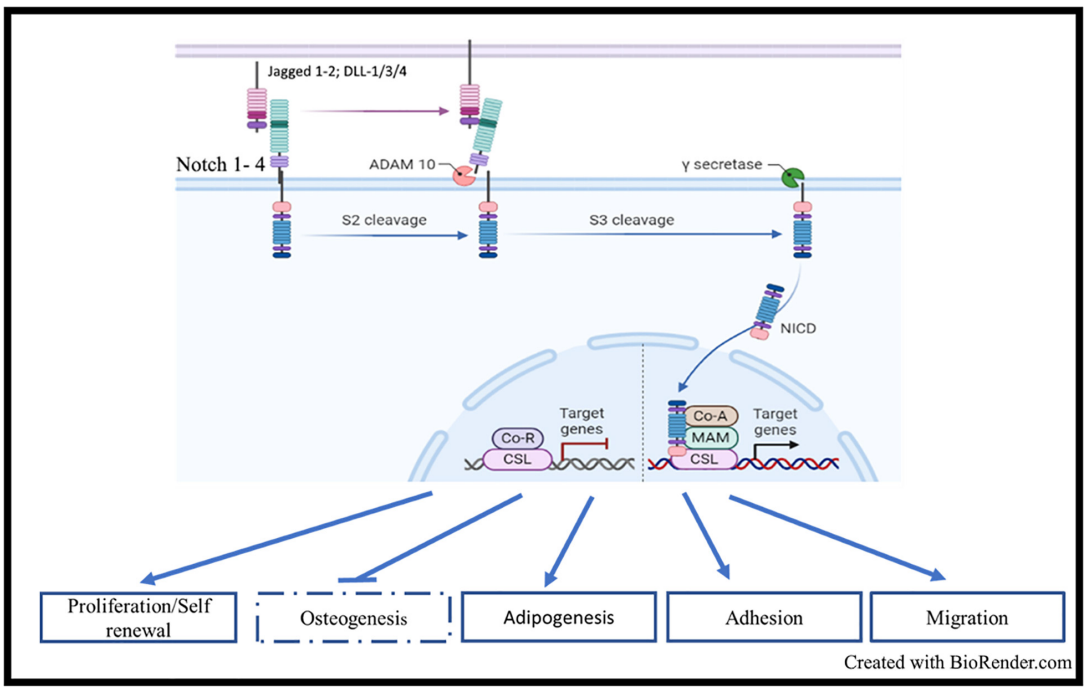

FIGURE 1 | Notch signaling, structure, and activation: Mammal Notch system involved 4 receptors (Notch 1, Notch 2, Notch 3, and Notch 4) and 5 ligands of the Delta/Serrate/lag-2 (DSL) [Delta-like ligands (DLL-1, 3-4), Jagged1, and Jagged2]. Interaction between ligand and receptor expressed on adjacent cells induces two proteolytic events S2 and S3, catalyzed by ADAM-like metalloprotease and gamma-secretase complex, respectively. These two proteolytic events lead to the release of the intracellular active form of the receptor, i.e., NICD. NICD enters into the nucleus and forms a transactivation complex in association with partners, such as Master-mind like-1 (MALM1), Recombining binding protein suppressor of hairless/Core Binding Factor-1, Suppressor of Hairless, Lag-2 (RBP-jk/CSL). This transcription complex promotes the expression of genes of the helix basic family, including *HES1*, *HEY1*, and many other genes such as *NF-κB*, *MYC* and *CCNDD*.

Fringe family and addressed at cell membrane, where it is then non-covalently associated as a single heterodimer, i.e., the Cterm corresponding to the PEST domain and the Nterm corresponding to the extracellular region. Interaction between ligand and receptor expressed on adjacent cells induces two proteolytic events S2 and S3, catalyzed by ADAM-like metalloprotease and gamma-secretase complex, respectively. These two proteolytic events lead to the release of the intracellular active form of the receptor, i.e., NICD (van Tetering and Vooijs, 2011). NICD enters into the nucleus and forms a transactivation complex in association with partners, such as Master-mind like-1 (MALM1), Recombining binding protein suppressor of hairless/Core Binding Factor-1, Suppressor of Hairless, Lag-2 (RBP-jk/CSL). This transcription complex promotes the expression of genes of the helix basic family, including Hes1, Hey1, and many other genes, such as NF-κB, Myc, and cyclin D, thus controlling cell proliferation, apoptosis, adhesion, invasion, and migration during development, organ patterning and developmental diseases (**Figure 1**; Gordon et al., 2008).

Notch Signaling in MSCs

As stemness signaling mediators, Notch components are expected to be present in MSCs (Moriyama et al., 2018). A comprehensive review of the literature reveals the presence of the transcript of all the four Notch receptors and ligands in MSCs (Zhang et al., 2019). Protein analysis through western immunobloting and flow cytometry supports the membrane expression of the four receptors. Western blot analysis showed that the proteins can be expressed as full length (220–280 kDa) transmembrane domains (90–110 kDa) (Takam Kamga et al., 2016a). Concerning ligands, most studies addressed the presence of Jagged1, while the expression of the other ligands are study-dependent. In general, DLL1, DLL-4, and Jagged 2 in less extend are reported, while a few studies support the expression of DLL-3. We observed that the expression of Notch ligands become readily detectable after 3 days of MSC culture (Kamdje et al., 2011), supporting the critical contribution of the physiologic state of MSCs when they are analyzed for Notch. In addition, MSCs in culture lose their stem cell-like properties after several subsequent passages; as Notch expression is negatively related to MSC senescence, cell passage should be considered when analyzing Notch expression (Mutyaba et al., 2014). Overall, MSCs express both Notch receptors and ligands, supporting the autocrine activation of Notch signaling. Nevertheless, mRNAs but not the related proteins of Notch target genes of the helix basic family, including Hes1, Hey, and He5 are represented in MSCs (Song et al., 2015). This observation is strengthened by the absence of cleaved form of Notch receptors in MSCs from healthy donors. Accordingly, MSC viability and differentiation are not affected by Notch pharmacological inhibitors, except for higher dose. It is unclear why the pathway is not active, regardless the presence of receptors, and ligands, but it is possibly

due to postrancriptional repression mechanisms. Lessons from developmental biology may shed some light. During tissue development, Notch signaling on adjacent cells is involved in a phenomenon of *trans*/*cis*-activation/inhibition called lateral inhibition/activation. This model supports the idea that during tissue specification, the activation/inhibition of the signaling occurs among adjacent cells with opposite fate, while the involvement of the pathway is poor among similar cells (Sato and Yasugi, 2020). Notch signaling is activated either as paracrine signal to mediate communication between two different cell types or as molecular event involving stem cells differentiation. The first involvement will be discussed in another section. Concerning Notch involvement in stem cell differentiation, osteoblast switch is the paradigm. Cao et al. observed that the Notch inhibitor DAPT or a specific Notch1 antagonist may reduce alkaline phosphatase (ALP) activity in MSCs undergoing BMP9-dependent osteoblast induction, thus leading to reduced osteogenic differentiation *in vitro* and *in vivo*. On the other hand, MSC treatment with DLL-1 enhances ALP, osteopontin (OPN) and osteocalcin (OCN) expression (Cao et al., 2017). Using lentiviral tools, Semenova et al. (2020) proposed that Notch-promoting osteogenesis is dose-dependent, because the pathway activation is required for the formation of osteoblasts, but higher activity of Notch leads to apoptosis. The involvement of Notch for osteoblast differentiation has been confirmed by many other studies. Cao et al. (2017) has stressed the specific involvement of Notch1 and DLL-1, but other receptors or ligands could participate to Notch activation during osteogenesis. Song et al. (2015) observed that adipocyte differentiation is associated with reduced expression of Notch signaling components, suggesting that Notch involvement during MSC differentiation is lineage-dependent, i.e., down-regulated for adipogenic differentiation and activated for osteogenic differentiation. This could be related to the tight crosstalk between Notch and BMP/Smad/runx2 signaling. Similarly, the involvement of Notch signaling in other MSC properties are mainly related to the crosstalk with specific signals. For example, through the stabilization of hypoxia-inducible factor 1 alpha (HIF-1α), hypoxia improves several MSC functions, including cell adhesion, migration, and proliferation. Ciria et al. (2017) observed that hypoxia upregulates the expression and activation of Notch signaling, while the absence of Notch signaling impairs HIF1α-induced MSC adhesion, migration, and proliferation. Lessons from hypoxia models have been very useful to understand that Notch can modulate almost all the MSC functions. Considering that Notch signaling is required for all these hypoxia-mediated events, we can therefore propose a model where the pathway itself is a pivotal signal required for all MSC features.

WNT SIGNALING IN MSCS

Wnt Signaling Structure and Activation

Wnt signaling is also an ancient and evolutionarily preserved pathway. Wnt proteins are secreted glycoprotein ligands that bind Frizzled transmembrane receptors located at cell membrane level. There are more than 19 Wnt proteins and 12 Frizzled receptors. There are two types of Wnt signaling pathway, the canonical Wnt/β-catenin cascade, and the non-canonical or β-catenin-independent signaling cascade (Kusserow et al., 2005). Initially, the ligands were classified as canonical (Wnt-1, −2, −3, −8a, −8b, −10a, and −10b) or non-canonical (Wnt-4, −5a, −5b, −6, 7a, −7b, and −11), according to the kind of signal activated upon their binding to the receptors (Siar et al., 2012). Some ligands indeed are more related to the type of activation (canonical or not), while some others can trigger Wnt signaling in a β-catenin-dependent or independent manner, according to the pathophysiological context. Wnt5a, for example, was early classified as non-canonical signal, but it can both activate and repress Wnt/β-catenin signaling during embryonic development and cancer development (Sato et al., 2010; van Amerongen et al., 2012). Studies on Wnt5a highlighted two important key points: i. the two cascades are not activated together, and ii. the co-receptors involved are different, i.e., ROR1/2 for the non-canonical signaling and the low-density lipoprotein receptor-related protein family (LRP5/6) for the β-catenin-related signal (Sato et al., 2010; van Amerongen et al., 2012). Indeed, Frizzled receptors are coupled to co-receptors, such as LRP5/6, ROR2, NRH1, Ryk, and PTK7. LRP5/6 is involved in the canonical signaling, where β-catenin is sequestrated by a destruction complex made of the Axin scaffold protein associated with APC (adenomatous polyposis coli), GSK-3β (glycogen synthase kinase 3β), and CK1 (casein kinase). CK1 and GSK-3β sequentially phosphorylate β-catenin at serines 45, 33, 37 or threonine 41 (Yost et al., 1996; Amit et al., 2002). This cascade of phosphorylation triggers ubiquitylation of β-catenin by βTrCP (an E3 ligase) and its subsequent proteasomal degradation. When the ligand binds to the frizzled receptors, its coreceptors LRP5/6 recruits the Disheveled (Dvl) protein, which in turn binds to Axin and GSK-3 proteins, leading to the disassembling of the destruction complex, the release of β-catenin and its nuclear localization (Salic et al., 2000). In the nucleus, β-catenin interacts with LEF/TCF transcription factors and other transcriptional activators to trigger activation of Wnt target genes (**Figure 2**). The canonical Wnt signaling can be modulated at different levels: (i) Inhibitors or antagonists of the ligand/receptors, such as Dickkopf (Dkk) proteins, secreted frizzled-related proteins (sFRPs), and WNT inhibitory factor 1 (WIF1); (ii) negative feedback through phosphorylation of Axins proteins (Axin 1 and Axin 2) by GSK-3β. There are several β-catenin-independent Wnt signaling pathways all related to a specific co-receptor or other key elements. One of them is the planar cell polarity (PCP) pathway that is mainly active in epithelial and mesenchymal cells, being involved in tissue polarization. The spatio-temporal organization of the pathway is not so clear; there are at least two complexes involved in Wnt-PCP located on adjacent cells, on the distal and the proximal membrane, respectively. Core components on the distal membrane consist in Frizzled and the scaffold partners Dvl, Diego and Flamingo. The counterpart on the proximal membrane involved Van Gogh, Prickle, and Flamingo scaffolds (Vladar and Königshoff, 2020). Although the two complexes are interconnected, a simple presentation of the signal transduction after ligand binding on Frizzled receptors shows the recruitment

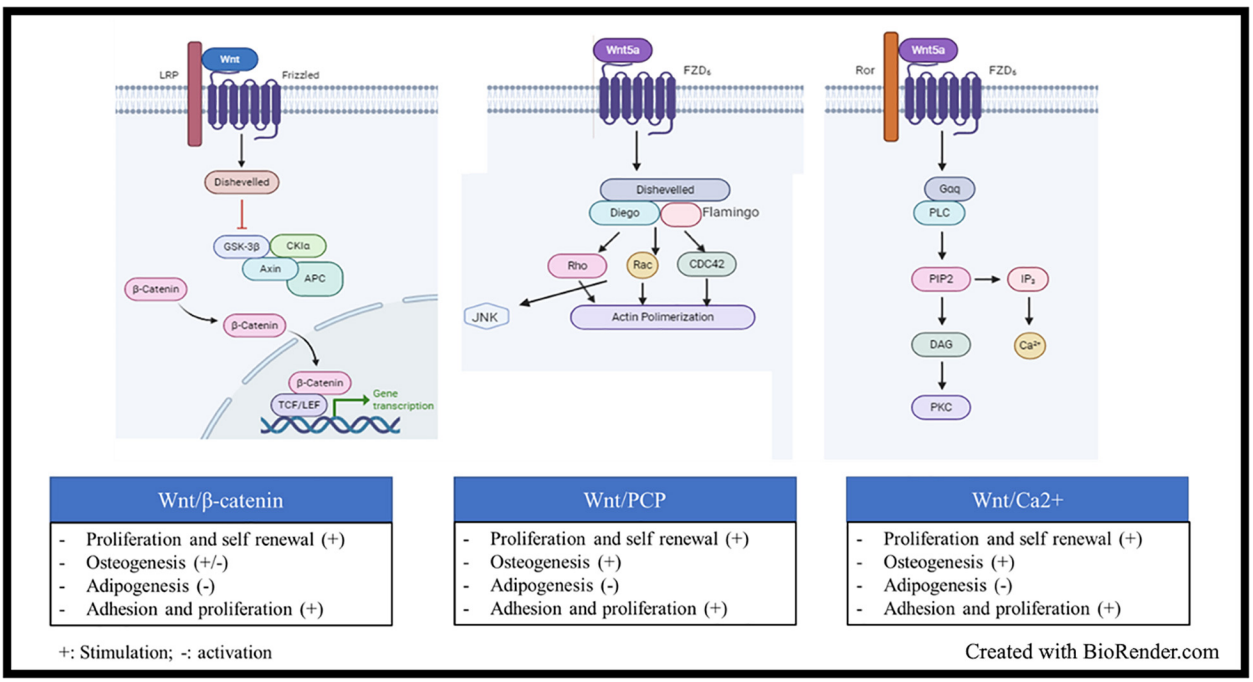

FIGURE 2 | Wnt signaling, structure and activation: Wnt signaling is activated when glycoprotein ligands of the Wnt family bind Frizzled transmembrane receptors located at cell membrane level. Upon ligand binding, several cascades could be activated: (i) The Wnt/β-catenin, the ligand binds to the frizzled receptors and its coreceptors LRP5/6, which recruits the Disheveled (Dvl) protein, which in turn binds to Axin and GSK-3 proteins, leading to the disassembling of the β-catenin destruction complex, the subsequent release of β-catenin and its nuclear localization In the nucleus, β-catenin interacts with LEF/TCF transcription factors and other transcriptional activators to trigger activation of Wnt target genes. (ii) The Wnt planar cell polarity (PCP) pathway, the ligand binding on Frizzled receptors shows the recruitment of Dvl, the scaffold proteins Diego and Flamingo and the formation of a protein platform triggering the activity of Rho family GTPase proteins to regulate actin organization and cytoskeleton dynamics. (iii) the Wnt/Ca^{2+} which controls the levels of intracellular Ca^{2+}. Upon ligand binding, Dvl is recruited and a G-coupled protein is also recruited, which subsequently activate the phospholipase C, whose role consists in the cleavage of phosphatidylinositol-4, 5-bisphosphate (PIP2) into inositol-1, 4, 5-trisphosphate (IP3) and diacylglycerol (DAG). The IP3 diffuses in the cytoplasm to induce Ca^{2+} release by cytoplasmic organelles. Ca^{2+} increase activates the Ca^{2+} -dependent kinases.

of Dvl, Diego and Flamingo and the formation of a protein platform triggering the activity of Rho family GTPase proteins to regulate actin organization and cytoskeleton dynamics (**Figure 2**; Siar et al., 2012; Vladar and Königshoff, 2020). Another well-known β-catenin-independent pathway is the Wnt/Ca^{2+}, which controls the levels of intracellular Ca^{2+} (**Figure 2**). Like the two afore mentioned cascades; Dvl is also recruited after ligand biding, but in the meantime a G-coupled protein is also recruited, which subsequently activate the phospholipase C, whose role consists in the cleavage of phosphatidylinositol-4,5-bisphosphate (PIP2) into inositol-1, 4, 5-trisphosphate (IP3) and diacylglycerol (DAG). The IP3 diffuses in the cytoplasm to induce Ca^{2+} release by cytoplasmic organelles. Ca^{2+} increase activates the Ca^{2+} -dependent kinases, such as protein kinase C (PKC), Calcium-calmodulin dependent kinase II (CamKII), and Calcium/calcineurin (CaCN). DAG also participates to the direct activation of PKC (Kusserow et al., 2005; Baksh et al., 2007; Jeong et al., 2020; Vladar and Königshoff, 2020).

The Wnt Signaling in MSCs

The role of Wnt signaling in the control of MSC biology is well documented. Transcriptomic and proteomic approaches, such as flow cytometry, ELISA, Western immunobloting, and mass spectrometry, showed in MSCs the enrichment in both canonical and non-canonical Wnt pathway components (Kuljanin et al., 2017). Using phosphospecific antibodies, we observed that Ser33/37/Thr41-phospho β-catenin (inactive) is totally absent in MSC cell lysate, thus suggesting that the Wnt/β-catenin is fully active in MSCs (Takam Kamga et al., 2016b; Wang et al., 2019). The requirement of a functional β-catenin-independent Wnt signaling, such as Wnt/Ca^{2+}, Wn/Jnk, Wnt/Ryk, Wnt/Ror2, was also described in MSCs (Qiu et al., 2011; Qu et al., 2013; Jeong et al., 2020). Overall, the activation of the pathway plays a critical role in cell fate decisions, notably for MSC proliferation, self-renewal and differentiation. In particular, Wnt signaling modulation in MSCs is widely investigated to fully exploit regenerative properties of MSCs in different research fields, such as bone, lung, and heart biology (Volleman et al., 2020). The canonical Wnt/β-catenin pathway sustains proliferation and renewal of MSCs; therefore, the use of pharmacological modulators of the pathway has brought several informations. The activation of the canonical Wnt/β-catenin pathway with lithium chloride or exogenous ligands, such as Wnt1 and Wnt3a, promotes MSC expansion by maintaining their clonogenic properties, but inhibits osteogenic, and adipogenic commitment (Liu et al., 2009, 2011; Jothimani et al., 2020).

One key mechanism of the suppressive role of Wnt/β-catenin on adipogenesis is the reduced expression of adipogenic transcription factors CCAAT/enhancer binding protein alpha (C/EBPalpha) and peroxisome proliferator-activated receptor gamma (PPARgamma) (Ross et al., 2000; Yuan et al., 2016). However, the use of Wnt/β-catenin inhibitors, such as Quercitin, reduce MSC proliferation and multipotency by favoring their osteogenic commitment and inhibiting both the chondrogenic and the adipogenic differentiation (Qu et al., 2013; Narcisi et al., 2015; Jothimani et al., 2020; Volleman et al., 2020). This model failed to explain the positive contribution of canonical Wnt in bone homeostasis *in vivo* (Wagner et al., 2020). Liu et al. suggested a role for Wnt/β-catenin activation levels; in fact, they observed a promoting effect with low concentrations of Wnt3a during osteogenic differentiation, through the regulation of key transcription factors such as RUNX2 and Osterix (Osx), while higher concentrations suppressed both osteogenesis and adipogenesis (Gaur et al., 2005; Liu et al., 2009). As for quercitin-mediating promotion of osteogenesis, increased Ca^{2+} signaling was also observed upon quercitin treatment, suggesting that osteogenic switch could be modulated by the balance between canonical and non-canonical signaling. In fact, a tight crosstalk between canonical and non-canonical Wnt leads to functional antagonism during osteogenic differentiation (Baksh et al., 2007), and osteogenic suppression induced by Wnt1 and Wnt3a is correlated with reduced Ror2/JNK levels (Gaur et al., 2005; Liu et al., 2009). Therefore, these studies proposed a binary view where the activation of Wnt/β-catenin through exogenous ligands, such as Wnt3a, may suppress both osteoblastic gene expression and MSC osteogenic differentiation with decreased matrix mineralization, while the activation of the non-canonical pathway has an opposite effects (Boland et al., 2004; Jothimani et al., 2020). Moreover, the activation of canonical pathway suppresses the non-canonical pathway and vice versa. Therefore, higher concentrations of Wnt3a suppresses osteogenesis by competing with non-canonical ligands. For instance, Wnt5a stimulates osteogenesis through the Wnt/ROR2/JNK signaling by competing with Wnt3a-mediated Wnt/β-catenin. Consequently, quercitin switches the balance toward non-canonical signaling, while Wnt3a or Wnt1 switch it toward Wnt/β-catenin cascade (Baksh et al., 2007). A role for canonical and non-canonical Wnt was also observed during motility and migration processes. Some authors used lentiviral constructs to enforce the expression of β-catenin or ROR2 in MSCs. They observed that β-catenin or ROR2 upregulation induces either nuclear β-catenin accumulation or the activation of Wnt5a/JNK and Wnt5a/PKC pathways, belonging to the canonical Wnt and non-canonical Wnt5a/ROR2 pathways, respectively (Liu et al., 2009; Cai et al., 2014).

MSC-DERIVED NOTCH AND WNT SIGNALING PATHWAYS IN HEMATOPOIESIS

Hematopoiesis is the process of blood cell formation through the proliferation and differentiation of HSCs and progenitor cells into specialized cells belonging to lymphoid and myeloid lineages (Orkin and Zon, 2008). Activation of Notch and Wnt signaling pathways is essential for the maintenance of HSCs (Bigas et al., 2010). Pharmacological and loss- or gain-of-function approaches have been useful strategies to investigate the role of Notch and Wnt signaling pathways in hematopoiesis. The retroviral expression in HSC/progenitors cell-enriched populations of active forms of Notch receptors, Notch target genes or β-catenin increases the pool of cells with repopulating capacities, such as Lin- cord blood cells, $CD34^+$ CD38- and mouse KLS (c-Kit^+ $Sca1^+$ Lin-) cells (Varnum-Finney et al., 2000; Kunisato et al., 2003; Reya et al., 2003; Vercauteren and Sutherland, 2004). Accordingly, the addition of exogenous ligands of the two pathways, such as Jagged-1 or DLL-1 (Notch signaling), and Wnt3a (canonical Wnt signaling), to cultures of purified primitive human blood progenitors induces self-renewal, survival and expansion of stem cells provided with pluripotent repopulating capacity in mouse models (Karanu et al., 2000; Willert et al., 2003; Delaney, 2005). Our and other groups have thoroughly described the expression of Notch and Wnt signaling in MSCs (Kamdje et al., 2011; Kamdje et al., 2012; Takam Kamga et al., 2016b), but other MSC-derived stromal components, including osteoblasts, and endothelial cells, can be the source of paracrine Wnt and Notch signaling in the BM (Nemeth et al., 2009; Wang et al., 2016). Moreover, MSCs can reconstitute the complete human BMME in irradiated mice (Muguruma et al., 2006) and therefore improve HSC engraftment following transplantation (Zhao et al., 2019). MSCs, expressing Notch and Wnt components, represent a major source of exogenous Notch or Wnt ligands that are involved in HSC fate. Using both co-culture and repopulation assay in SCID mice, Kadekar et al. observed that MSCs supported HSC expansion by preventing the apoptosis of primitive HSCs through a higher expression of β-catenin, DLL-1, Jagged1, Hes1, Notch1, and cleaved Notch1 (NICD1) (Kadekar et al., 2015). Similarly, several works have clearly showed the enhanced expression of Notch and Wnt signaling in both co-cultured MSCs and hematopoietic progenitors leading to proliferation and maintenance of HSCs on MSC feeder layer (Kim et al., 2009, 2015a, 2018; Kikuchi et al., 2011). Interestingly, increased levels of Notch components in MSCs resulted from the activation of β-catenin pathways. Growing evidence supports a model where HSC-MSC co-culture leads to higher level of β-catenin in MSCs, whose gene transactivation may lead to Jagged1 expression, which in turn acts as paracrine ligand to trigger activation of Notch signaling in HSCs. Wnt/β-catenin signals in MSCs enhance HSC self-renewal by inducing the crosstalk of Wnt-Notch signals in the HSC niche (Kim et al., 2009; Oh, 2010; Kadekar et al., 2015). Therefore, the canonical Wnt signaling is significantly required by stromal cells (Jeong et al., 2020). Excess of canonical Wnt signaling in HSCs impairs the function of HSCs and their multilineage progenitors (Scheller et al., 2006); as previously mentioned, this could be explained by the competition between canonical and non-canonical Wnt cascades. Higher levels of canonical signaling suppress the non-canonical one. Activation of the non-canonical Wnt, with Wnt5a and the co-receptor Ryk, leads to HSC quiescence, whereas Wnt3a,

the canonical ligand, supports HSC proliferation (Liu et al., 2011; Jeong et al., 2020). The involvement of the non-canonical cascade may explain why Notch and Wnt pathways are also involved in mediating adhesion and migration of HSCs. The aforementioned work by Kadekar et al. showed enhanced levels of Wnt/Notch components as well as migration and adhesive properties in HSCs cultured on MSCs (Kadekar et al., 2015). The crosstalk of Notch or Wnt pathways with stromal cell-derived factor-1 (SDF-1)/CXCR4 axis is well described and may be responsible for their influence on HSCs migration and adhesion (Tamura et al., 2011; Kadekar et al., 2015). Duryagina et al. (2013) observed that Jagged1 expression by MSCs induces the release of SDF-1, thus supporting proliferation, migration, and adhesion of $CD34^+$ progenitors, resulting in the increase of cobblestone area-forming cells and long-term culture-initiating cells (LTC-ICs). Notch and Wnt signaling are involved not only in the maintenance of HSCs, but also in T-cell differentiation. Delaney et al. observed that the treatment of $CD34^+$ $CD38^-$ cord blood progenitors with low density of DLL1 enhanced generation of NOD/SCID repopulating cells, while high density of DLL1 induced a switch toward lymphoid rather than myeloid lineage (Delaney, 2005). However, higher levels of Notch pathway preferentially support T cell differentiation by stimulating the common lymphoid progenitor toward T-cell rather than B-cell lineage. Precursor cells engineered to express NICD1 and engrafted in immunodeficient mice give rise to T-cell populations only. Conversely, silencing Notch activity leads to the onset of B-cell progeny (Wilson et al., 2001). Similarly, MSCs may support T-cell differentiation of co-cultured precursor cells when forced to express Notch receptors (Notch1 and Notch2) and ligands (Jagged1 and DLL1) (Felli et al., 1999; Aster, 2005; Vacca et al., 2006). During this process, the type of the ligands expressed by stromal cell is crucial. Some MSC cell lines, such as OP9, expressing different Notch ligands, showed that MSC-derived DLL4 supports both αβ- and γδ-lineage differentiation, while MSC-derived Jagged1 supports TCR-αβ, but not TCR-γδ development and MSC-derived Jagged2 mainly supports γδ T cell differentiation at the expense of αβ T cells (Van de Walle et al., 2013). Assays with OP9 cell line were also useful to understand the contribution of stromal cell-derived Wnt signaling to T-cell development. Famili et al. (2015) engineered OP9 cells to conditionally express either Wnt3a or Wnt5a. They observed that low density of the canonical Wnt ligands accelerates T-cell proliferation and maturation, while higher levels of the signal blocks T-cell development and favors alternative lineages. In parallel, *in vitro* experiments showed no effect of the non-canonical Wnt ligand (Wnt5a). During the T-cell switch, thymic stromal cell-derived Wnt signaling influence T-cell expansion and maturation by controlling the activation of transcription factors of the T-cell factor/lymphoid enhancing factor (Tcf/Lef) family (Schilham et al., 1998; Staal et al., 2001; van Loosdregt et al., 2013). This is associated with defective final differentiation and reduced thymocyte number in mice, either expressing the inhibitor of β-catenin and Tcf (ICAT) or resulting deficient for canonical Wnt ligand, such as Wnt1 (Mulroy et al., 2002; Pongracz et al., 2006). Famili et al. (2015) observed that in the co-co-culture setting with OP9 cell line or in mouse models, low levels of β-catenin signaling supports T-cell development, whereas higher activity of canonical and non-canonical Wnt preferentially favors myeloid and B-cell developments. Notably, the regulation of hematopoiesis by canonical Wnt requires the physical contact between MSCs and hematopoetic cells (Ichii et al., 2012; Famili et al., 2015). MSCs and stromal cell mediated Wnt signaling is therefore required at all steps of the hematopoiesis, being a decisional factor for lymphoid and myeloid switch. Concerning myeloid lineage, the role of Notch and Wnt pathways is not well-defined compare to the lymphoid counterpart. For example, myelopoiesis has been associated with low levels of Notch signaling (de Pooter et al., 2006; De Obaldia et al., 2013). However, this view may underestimate the complexity of Notch contribution to myeloid lineage development. Notch involvement in myeloid differentiation is certainly lower, as compared to lymphopoiesis (De Obaldia et al., 2013); nevertheless, the fine tuning of Notch levels is fundamental for myeloid cell development. The role of Notch could be phase-dependent during myeloid cell generation (Fehon et al., 1991). For instance, constitutive Notch activation in 32 myeloid progenitor cells led to self-renewal of myeloid precursors and inhibition of granulocytic differentiation (Milner et al., 1996). The same results were also achieved in HL-60 cell line, which failed to undergo ATRA-mediated differentiation when genetically enforced to express NICD1 (Carlesso et al., 1999). Conversely, Jagged1 may inhibit proliferation of macrophage progenitors (Masuya et al., 2002; Kim et al., 2009; Kadekar et al., 2015) and Notch pathway seems to be involved in the differentiation of mature myeloid cells (Fehon et al., 1991). The complexity of Notch contribution to myeloid lineage could arise from the level of the pathway activation. Using *ex vivo* systems for the expansion of cord blood CD34 + CD38- HSC progenitors, DLL-1 at lower density was capable of enhancing the generation of $CD34^+$ cells as well as $CD14^+$ and $CD7^+$ cells, consistently with early myeloid and T-cell differentiation, respectively. However, culture with higher amounts of DLL-1 induced apoptosis of $CD34^+$ precursors, thus resulting in decreased cell numbers, without any effects on the generation of $CD7^+$ cells (Delaney, 2005). A minimal activity of Notch could be necessary for the maintenance of myeloid progenitors, while higher activation could induce cell differentiation. Again, the source of paracrine ligands that trigger Notch activation in myeloid progenitors might be stromal cells. Indeed, primitive ($CD34^+$ $CD38^-$ Lin^-), and intermediate ($CD34^+$ $CD38^+$ Lin^-) HSCs cultured on MSCs expressing Jagged1 or DLL-1 showed enhanced self-renewal properties associated with increased expression and activation of Notch1. This suggests that in the BM niches MSCs provide exogenous Notch ligands necessary for the maintenance of myeloid progenitor pool and Jagged1 expression is the consequence of Wnt/β-catenin activation, thus suggesting a role for Wnt-Notch cross-talk in myelopoiesis (Fernández-Sánchez et al., 2011). In parallel, thanks to *in vitro* colony-replating assays, Nteliopoulos et al. observed that canonical and non-canonical Wnt-3 can stimulate proliferation of myeloid progenitors and impair IL-3-induced differentiation into myeloid populations (Nteliopoulos et al., 2009). As MSCs are a source of Wnt ligands, we can

hypothesize that stromal cells may support the self-renewal of myeloid progenitors through the release of Wnt ligands (Toni et al., 2006). However, there are a few studies addressing the role of MSC-derived Wnt signaling in myeloid counterpart. Most data arise from studies on myeloid malignancies and will be discussed in the next section.

MSC-DERIVED NOTCH AND WNT SIGNALING IN LEUKEMIA

Notch in Leukemia

Several studies have addressed the role of Notch in leukemic diseases (**Table 1**). Early association between Notch and hematopoietic malignancies was shown in T-ALL, where more than 50% of patients have activating mutations of Notch signaling, thus representing the first gene aberration in T-ALL (Weng et al., 2004). Notch mutations in T-ALL mainly target the HD or the PEST domains. By sequencing the heterodimerization domain of NOTCH1 in mouse models of T-ALL, O'Neil (2006) found that more than 74% of the tumors harbored activating mutations in Notch1. Mutations in HD domain induce a constitutive, ligand-free activity of the receptors. The second hotspot of mutations is the PEST domain targeting NICD to ubiquitination-mediated proteolysis. The mutation in the PEST domain determines the lack of degradation of the active form of the receptors, thus leading to a constitutive activity of the pathway (Weng et al., 2004). In nude mouse models of T-ALL, tumor establishment correlated with Notch1 mutation (Lin, 2006). The importance of Notch activation for T-ALL cell survival has raised the use of gamma-secretase inhibitors (GSIs). T-ALL cells are highly sensitive to different GSIs (Grosveld, 2009; Real and Ferrando, 2009; Baratta, 2019) as well as to other Notch inhibitors, such as Notch transcription factor inhibitors (Moellering et al., 2009) and Notch blocking antibodies (Wu et al., 2010). Besides Notch1, higher levels of Notch3 were found in T-ALL cells, and its genetic inhibition through siRNA led to growth inhibition and apoptosis (Masiero et al., 2011). Constitutive activation of Notch is also a hallmark of B-cell CLL. Notch activating mutations occur essentially in the PEST domain of Notch receptors and are associated with a shorter overall survival (Willander et al., 2013). Rosati et al. (2013) found high expression of Notch1, Notch2, Jagged1, and Jagged2 in CLL correlated with higher activation of the pathway. This activation is further increased in CLL cells that are resistant to spontaneous apoptosis in *ex vivo* culture. Accordingly, our group demonstrated that Notch inhibition, through GSIs or blocking antibodies, induces CLL apoptosis, and sensitizes leukemia cells to treatment with chemotherapeutic agents (Kamdje et al., 2012). Except in T-ALL, Notch mutations are very rare in other leukemia types, where its role is either well defined or quite controversial (Liu et al., 2013). In B-cell acute lymphoblastic leukemia (B-ALL), Notch1 mutation was not observed, but a tumor suppressor role of the pathway was suggested (Morimura et al., 2000; Zweidler-McKay et al., 2005). Notch seems to be epigenetically silenced in B-ALL, since Notch3, Jagged1, Hes2, Hes4, and Hes5 are frequently hypermethylated in leukemia B-cell lines and primary B-ALL cells. Restoration of Hes5 expression by lentiviral transduction resulted in growth arrest and apoptosis in Hes5-negative B-ALL cells (Kuang et al., 2013). Activation of the pathway induces growth arrest and apoptosis in B-ALL cells (Morimura et al., 2000; Zweidler-McKay et al., 2005; Kuang et al., 2013). Putting in the context of anti-leukemic treatment, epigenetic analysis of blast cells collected from B-ALL patients along the course of the disease revealed that the methylation pattern of Notch receptors' genes changes according to the disease step. It was observed that Notch genes receptors are highly methylated at diagnosis, less methylated upon drug treatment and became hypermethylated in relapsed patients (Takam Kamga et al., 2019a). These observations suggested that the methylation status of Notch genes might be relevant for drug response. This is strengthened by the results obtained in non-leukemic systems where evidence of epigenetic modulation of Notch genes in cancer cells treated with chemotherapeutic agents like 5-fluorouracil and cisplatin was demonstrated (Maeda et al., 2014). Collectively these data support further research to unravel the role of epigenetic silencing of Notch in leukemia disease. Studies in solid cancers have also reported that Notch genes are the targets of several miRNA (or vice-versa) involved in drug resistance including miR-1, miR-200, miR-34 etc. (Ji et al., 2009; Li et al., 2009). Consistently recent studies have provided the evidence that the BM-microenvironment transfer miRNA in leukemia cells, supporting cell survival (Liu et al., 2015; Ganesan et al., 2019).

Our group has recently shown that human BM MSCs, through Notch activation, protect B-ALL cells from apoptosis induced by chemotherapeutic agents; in fact, Notch signaling inhibition abrogates the protective role of human BM MSCs toward B-ALL cells (Kamdje et al., 2011), thus highlighting the contribution of the BMME in Notch signaling. In myeloid malignancies, the role of Notch is still matter of investigation. In chronic myeloid leukemia (CML), Notch emerges as tumor suppressor gene rather than oncogene, although still poorly investigated. Yin et al. (2009) observed that overexpression of Notch1 active form in the CML cell line K562 significantly inhibits cell proliferation, while knocking-down the pathway through the expression of a dominant negative of RBP-jk promotes colony-forming activity. In acute myeloid leukemia (AML), the role of Notch remains controversial: Kannan et al. (2013) described Notch expression and activation in *ex vivo* AML cell samples and AML cell lines, but weak activation of the pathway, as demonstrated by the low expression level of the Notch target genes. Similarly, Lobry et al. (2013) described epigenetic silencing of Notch target genes in AML; consistently, they demonstrated that the reactivation of Notch signaling induced apoptosis and differentiation of leukemia blast cells into mature cells. These results are consistent with the anti-leukemic role of demethylating/hypomethylating agents azacytidine or decitabine in AML (DiNardo et al., 2018; Leung et al., 2019). However, our and other groups found that Notch activation is not homogenous within AML samples and cell lines (Tohda and Nara, 2001; Sliwa et al., 2014; Czemerska et al., 2015). In the study by Tohda and Nara (2001) 6 cell lines out of 8 and 40% of AML fresh samples showed active forms of Notch1

TABLE 1 | Roles of Notch and Wnt signaling pathways in leukemia.

		Leukemia cell-derived Notch/Wnt signaling	MSC-derived Notch/Wnt signaling
AML	Biomarkers	– Higher expression and activation of Notch signaling components is associated to poorer prognosis in AML (Xu et al., 2011; Sliwa et al., 2014; Takam Kamga et al., 2019a). – High activation of Wnt/β-catenin is associated to shorter survival (Khan and Bendall, 2006; Griffiths et al., 2010).	– Overexpression of Notch1 and Jagged1 in AML-MSCs (Takam Kamga et al., 2016a). – Overexpression of Wnt molecules in AML-MSCs (Takam Kamga et al., 2016b).
	Oncogene	– Notch/Jagged1 expression and activation in acute promyelocytic leukemia (APL) supports leukemia cell growth (Grieselhuber et al., 2013). – Activation of Wnt/β-catenin/TCF/LEF pathway supports growth of leukemia cells (Khan and Bendall, 2006). – Epigenetic modification of Wnt inhibitors in AML (Griffiths et al., 2010).	– Notch signaling is required for β-catenin-mediated oncogenesis in mouse models of AML (Kode et al., 2014). – MSC-derived Notch signaling supports growth and survival of leukemic cells (Takam Kamga et al., 2016b). – MSC-derived Notch signaling supports growth and survival of leukemic cells (Takam Kamga et al., 2016a).
	Tumor suppressor	– Enforced expression of Notch receptors in AML inhibits leukemia cell growth and survival (Kannan et al., 2013; Lobry et al., 2013)	
	Mediator of drug resistance		– MSC-derived Notch signaling reduces apoptosis in AML treated with chemotherapeutic agents (Takam Kamga et al., 2016a). – Stromal cell-derived Wnt signaling reduces apoptosis in AML treated with chemotherapeutic agents (Takam Kamga et al., 2016b)
B-ALL	Biomarkers	– Higher expression and activation of Notch signaling is observed in refractory patients (Kamdje et al., 2011; Takam Kamga et al., 2019b). – Wnt ligands and receptors are overexpressed in B-ALL cells (Khan et al., 2007). – Overexpression of LEF1 predicts poor outcomes (Kühnl et al., 2011)	
	Oncogene	– Epigenetic inactivation of Notch in B-ALL (Kuang et al., 2013). – Stimulation of Wnt/β-catenin signaling supports growth and survival of B-ALL cells (Khan et al., 2007).	– MSC-derived Notch signaling supports growth and survival of leukemic cells (Kamdje et al., 2011). – MSC-derived Wnt signaling supports growth and survival of leukemic cells (Yang et al., 2013).
	Tumor suppressor	– Activation of Notch signaling induce cell cycle arrest and apoptosis (Morimura et al., 2000; Zweidler-McKay et al., 2005; Kuang et al., 2013).	
	Mediator of drug resistance	– Notch inhibitors sensitize B-ALL cells to chemotherapy (Takam Kamga et al., 2019b). – Wnt inhibition sensitizes B-ALL to chemotherapy (Fu et al., 2019).	– MSC-derived Notch signaling reduces apoptosis in B-ALL treated with chemotherapeutic agents (Kamdje et al., 2011). – MSC-derived Wnt signaling reduces apoptosis in B-ALL treated with chemotherapeutic agents (Yang et al., 2013).
CLL	Biomarkers	– Notch activating mutation are observed in CLL patients (Willander et al., 2013). – Notch1 mutation is found in intermediate-risk patients, predicting poorer survival (Willander et al., 2013). – Higher expression and activation of Notch signaling is observed in refractory patients (Rosati et al., 2013). – Wnt5 is enriched in CLL patients (Janovska et al., 2016). – Low WNT3 expression is a signature of patient with short therapy-free survival (Janovská and Bryja, 2017).	
	Oncogene	– Activation of Notch signaling supports growth and survival of CLL cells (Kamdje et al., 2012; Rosati et al., 2013). – Lef1 is a prosurvival factor s (Willander et al., 2013). – Wnt/PCP controls migration of CLL cells (Janovska et al., 2016).	– MSC-derived Notch signaling supports growth and survival of leukemic cells (Kamdje et al., 2012). – MSC-induced accumulation of β-catenin in CLL cell supports growth and survival of leukemia cells (Mangolini et al., 2018).
	Tumor suppressor		
	Mediator of drug resistance	– Notch inhibitors sensitize CLL cells to chemotherapy (El-Gamal et al., 2014).	– MSC-derived Notch signaling reduces apoptosis in CLL cells treated with chemotherapeutic agents (Kamdje et al., 2012; Mangolini et al., 2018). – MSC-induced accumulation of β-catenin in CLL cells, supports drug resistance of leukemia cells (Mangolini et al., 2018).

(Continued)

TABLE 1 | Continued

		Leukemia cell-derived Notch/Wnt signaling	MSC-derived Notch/Wnt signaling
CML	Biomarkers		
	Oncogene	– β-catenin is a target of BCR-ABL (Zhao et al., 2007; Tomasello et al., 2020) – Wnt1 signaling supports growth and survival of CML cells (Majeti et al., 2009).	
	Tumor suppressor	– Notch1 suppresses growth and survival of K562 cell line (Yin et al., 2009).	
	Mediator of drug resistance	– Inhibition of Wnt/β-catenin sensitizes cells to TKI (Zhang et al., 2013).	– MSC-derived Notch signaling reduced apoptosis in CML treated with chemotherapeutic agent. – MSC-derived Wnt signaling reduced apoptosis in CML cells treated with TKI (Han et al., 2013; Zhang et al., 2013).
T-ALL	Biomarkers		
	Oncogene	– Notch1 is mutated in more than 50% of patients (Weng et al., 2004). – Notch signaling drives oncogenesis and supports growth and survival of T-ALL cells (Weng et al., 2004; O'Neil, 2006). – Notch 3 supports survival of T-ALL cells (Masiero et al., 2011).	
	Tumor suppressor		
	Mediator of drug resistance	– Notch inhibition sensitizes cells to drug treatment (Grosveld, 2009; Real and Ferrando, 2009).	– MSC-derived Notch/Jagged1 signaling reduces apoptosis in Jurkat cell line treated with chemotherapeutic agents (Yuan et al., 2013). – MSC-derived Wnt signaling reduces apoptosis in ALL cell treated with chemotherapeutic agents (Yang et al., 2013).

receptors. Some observations suggest that Notch expression and activation levels in AML could be correlated with the molecular background of each samples or the FAB subgroup (Tohda and Nara, 2001; Salat et al., 2008; Grieselhuber et al., 2013; Sliwa et al., 2014; Czemerska et al., 2015; Takam Kamga et al., 2019a). For example, ETO in association with RBP-jk inhibits the expression of Notch target genes, while the leukemogenic fusion protein AML1/ETO is devoid of this repressive activity (Salat et al., 2008). Grieselhuber et al. (2013) identified Notch expression and activation in acute promyelocytic leukemia presenting the PML-RARα rearrangement. However, Notch pathway activation has been observed mostly in more immature AML subtypes and was associated with bad prognosis, as patients with hyper-expression of Notch1 displayed poorer overall survival (Xu et al., 2011; Sliwa et al., 2014; Takam Kamga et al., 2019a). Notably, in a recent study we found that less mature AML subtypes (M0-M1) expressed high levels of all the four receptors (Notch1–4) and some ligands (Jagged2, DLL-3), whereas adverse cytogenetic risk groups overexpressed Notch3, Notch4, and Jagged2 as compared to good cytogenetic risk patients. Accordingly, univariate and multivariate analysis confirmed a longer overall survival for patients presenting low expression of Notch4, Jagged2, and DLL3 on leukemia cells at diagnosis (Takam Kamga et al., 2019a).

Wnt Signaling in Leukemia

Wnt pathway deregulation is a common feature of leukemia. In lymphoid malignancies, such as ALL, CLL non-canonical and canonical Wnt pathway-related genes and proteins are over-expressed in lymphoid tumor cells, thus resulting prone to apoptosis upon interference with the pathway including β-catenin inhibition (Rosenwald et al., 2001; Lu et al., 2004; Janovská and Bryja, 2017). Consistently, over-expression of LEF-1 mRNA is a hallmark in ALL and CLL patients with poor prognostic. The constitutive activation of the pathway deregulation can result from gene mutation (Tomasello et al., 2020), but also from epigenetic modifications. In CLL for example, Next generation sequencing of samples from patients confirmed that 40% of patients harbors somatic mutations in Wnt pathway components (*WNT1*, *WNT10A*, *DKK2*, *RSPO4, FZD5, RYK*) (Wang et al., 2014). Studies have indicated a crosstalk between molecular aberrations and epigenetic activation of the pathway, acting in a concerted manner to interfere with Wnt inhibitors while promoting Wnt agonists or activators. Consistently the promoter of genes coding for Wnt pathway inhibitors including *WIF1, DKK3, APC, SFRP1, SFRP2, SFRP4,* and *SFRP5* are frequently hypermethylated and consequently downregulated in samples from CLL and ALL (Roman-Gomez et al., 2004; Martin et al., 2008; Rahmatpanah et al., 2009). It is worthy to mention that the tumor suppressor gene *APC* could also be the target of epigenetic modification. In T-ALL, the promoter of APC is methylated in about 50% of cases and correlates with β-catenin over-expression (Matsushita et al., 2006). In B-ALL cell lines and primary B-ALL cells, the Wnt pathway is activated by over-expression of Wnt proteins and receptors (Wnt-2b, Wnt-5a; Wnt-10b, Wnt-16b; FZD7; FZD8) and their stimulation with Wnt-3a increases the survival and proliferation of these cells (Khan et al., 2007). Similarly to what is observed in CLL, the hyperactivation of the pathway is due at least in part to the hypermethylation

of the Wnt inhibitors (Kong et al., 2018). Concerning myeloid malignancies, Zhao et al. found that β-catenin deletion causes a reduction in the ability of mice to develop BCR-ABL-induced CML (Zhao et al., 2007). Indeed, stabilization and nuclear localization of β-catenin is a direct consequence of the BCR-ABL (Tomasello et al., 2020). As a consequence, the treatment of CML stem/progenitor cells with β-catenin inhibitor ICG001 reduces cell survival and proliferation by sensitizing cells to tyrosine kinase inhibitors (TKI). Interestingly, the addition of purified Wnt1 activates β-catenin and protects CML cells from TKI treatment, thus confirming the important role of Wnt pathway in maintaining CML stem cells (Zhang et al., 2013). In AML, our and other groups have observed an enrichment in Wnt components in AML primary cells compared to normal hematopoietic progenitors, although the expression of the Wnt components was not homogenous across samples (Majeti et al., 2009). Interestingly, β-catenin was enriched in high-risk patients; subsequently, we observed that patients presenting higher activation of the pathway had shorter progression free survival (Takam Kamga et al., 2020). The pivotal role for Wnt pathway in AML pathogenesis is also supported by studies in which cells transfected with AML-associated translocation products (PLZF-RARA and AML1-ETO) display activation of pakoglobin, a homolog of β-catenin. This induction is followed by the transactivation of TCF/LEF transcription factors and the increase in the proliferation and survival of murine hematopoietic progenitor cells (Khan and Bendall, 2006; Griffiths et al., 2010). In fact, the constitutive activation of Wnt signaling in AML may not result from β-catenin mutation, but from Flt3 hyperexpression leading to Akt-mediated phosphorylation and GSK-3β inactivation, with β-catenin stabilization (Brandts et al., 2005; Román-Gómez et al., 2007; Valencia et al., 2009). In accordance with the pro-oncogenic role of Wnt in AML, β-catenin down-regulation in AML cell lines and *ex vivo* cells through shRNA or pharmacological inhibitors, such as quercitin IWP-2, Niclosamide and PNU-74654, decreases their proliferation rate *in vitro* and homing as well as their engraftment after xenotransplantation (Toni et al., 2006; Gandillet et al., 2011; Takam Kamga et al., 2020). Interestingly, the Wnt inhibitors quercetin induced pronounced apoptosis in AML, *in vivo* and *in vitro* in part by its demethylating activity (Maso et al., 2014; Alvarez et al., 2018). In fact, in AML, the use of demethylating agents such as Decitabine decreased methylation status of Wnt antagonist including SFRP1, HDPR1, and DKK3, providing evidence that activation of the pathway resulted from an epigenic silencing (Li et al., 2014). Similarly to CLL, in AML the promoter of genes coding for Wnt antagonists (sFRP1, sFRP2, sFRP4, sFRP5, DKK1, and DKK3 etc.) are frequently methylated predicting poor outcome in patients (Jost et al., 2008; Valencia et al., 2009).

The Role of MSCs in Leukemia

As previously discussed, several studies have reported a supportive and protumorigenic role for MSCs toward different leukemia subtypes, including AML, B-ALL, CLL, CML, and T-ALL (Lee et al., 2019). A comparison of MSCs isolated from myeloid and lymphoid leukemia environment compared to MSCs isolated from healthy donors revealed that stromal cells are the sites of deep molecular changes involving modulation of the expression and/or secretion of cytokines, chemokines, adhesion molecules, and extracellular matrix molecules such as SDF-1/CXCR4, CD44. These modifications are thought to improve MSCs-mediated survival and growth of leukemic cells and mainly leukemia stem/progenitors cells (Ge et al., 2011; Yu et al., 2019; Azadniv et al., 2020). MSCs have the double ability to keep leukemic stem cells in a quiescent state while promoting proliferation and growth of leukemia cells. Coculture experiments showed that MSCs supports the culture of primary leukemia cells and promote the long term survival of leukemia stem cells (Ito et al., 2015). Evidence from studies support a bidirectional crosstalk between MSCs and leukemia stem/progenitor cells. In the study of the Yu et al. (2019), they observed that MSCs co-cultured with B-ALL leukemia stem cells showed downregulation of lumican increased expression of CD44 and diverse chemokine including IL-3, IL-7, IL-10, and G-CSF. These educated MSCs were more potent to protect leukemic cells against VP-16. Similarly, in AML, CXCR4, CD44, integrins like VCAM1 or VLA-4 are activated upon the contact between AML cells and MSCs to promote resistance of leukemia cells. A treatment of AML cells with the specific CXCR4-SDF inhibitor, AMD3100 or antibodies against CD44, VCAM1, significantly sensitizes AML stem cells to treatment with chemotherapeutics, thus abrogating MSCs mediated chemoresistance and persistence of the minimal residual disease (Matsunaga et al., 2003; Tabe et al., 2007; Nervi et al., 2009; Jacamo et al., 2014). This mechanism can be translated in other leukemia as demonstrated by several studies (Konopleva et al., 2009).

Putting Together the Contribution of MSC-Derived Notch and Wnt Signaling Pathways in Leukemia

Stromal BMME promotes the survival of leukemia cells through the activation of many pathways, including Notch and Wnt signaling (Vianello et al., 2010; Kamdje et al., 2011, 2012; Tabe and Konopleva, 2015; Cai et al., 2016; Takam Kamga et al., 2016a). On the other hand, Notch and Wnt signaling are the targets of persistent modifications occurring often in parallel in the BM niche during leukemogenesis (Kode et al., 2014; Kim et al., 2015b). Therefore, analyzing MSCs isolated from leukemia samples can provide an overview of these persistent modifications involving both pathways, which eventually can be considered as a unique microenvironmental communication system, the so called Wntch pathway (Sengupta et al., 2007; Hayward et al., 2008; Takam Kamga et al., 2016a; Azadniv et al., 2020). Studies revealed that, increasing activity of Notch signaling results from an aberrant β-catenin signaling in the same stromal compartment and vice versa (Kode et al., 2014). In normal hematopoiesis, stromal β-catenin signaling induces expression of Jagged1; consequently, stromal Jagged1, and Wnt ligands induce in HSCs Notch and Wnt signaling, respectively, and support their self-renewal in a cell-to-cell contact-dependent manner (Ichii et al., 2012; Kadekar et al., 2015). The same phenomenon occurs in leukemia cells and stem cells, where studies reported higher levels of stromal Notch parallel with higher activation of the Wnt signaling

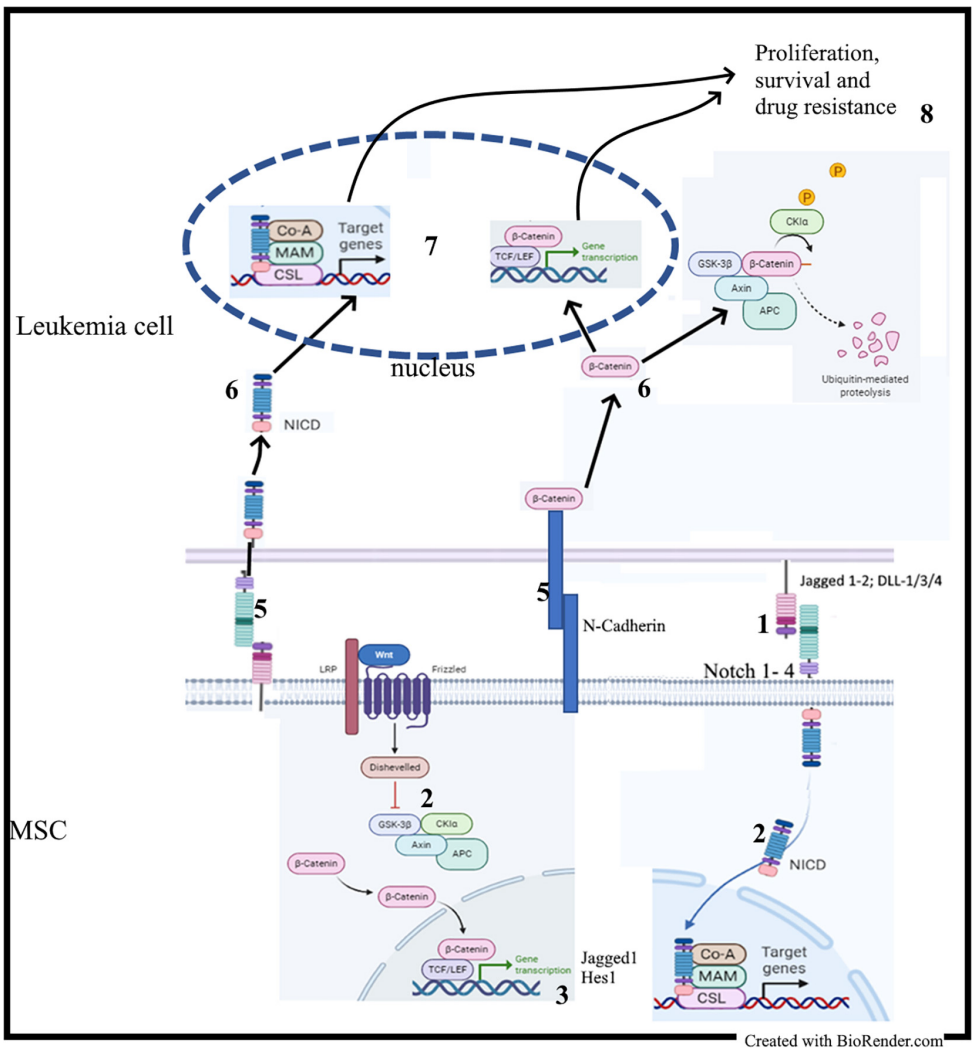

FIGURE 3 | MSC-derived Notch and Wnt signaling in leukemia: (1) Contact between MSC and leukemia cells; (2) Activation of the Notch and Wnt signaling cascades in MSC; (2) Synthesis of Notch and Wnt target genes including Jagged1; (4) Upregulation of Notch1 and Jagged1 expression; (5) Activation of adhesion molecules and Notch signaling; (6) Release of NICD and stabilization of β-catenin; (7) Transactivation of Notch and Wnt target genes.

(**Figure 3**) (Yang et al., 2013; Takam Kamga et al., 2016a,b). Therefore, Notch signaling is required for leukemic role of the canonical Wnt (Kode et al., 2014). The functional outcome of this Wnt/Notch crosstalk between MSCs and B-ALL or AML cells is the induction of leukemia cell proliferation, survival and chemoresistance. Consequently, Wnt and/or Notch inhibition through pharmacological modulators, including small molecules inhibitors (PNU-74654, Niclosamide, GSIs) and Notch blocking antibodies, may sensitize leukemia cells to drug treatment, thus abrogating the protective role of MSC monolayer (Kamdje et al., 2011; Takam Kamga et al., 2016a, 2020; Fu et al., 2019). This antileukemic role requires the production of reactive oxygen species (ROS) and the modulation of prosurvival proteins, such as mTor, NF-κB, STAT-3, and Erk (Kamdje et al., 2011; Takam Kamga et al., 2016a,b). This role observed in *ex vivo* co-culture systems was validated in mouse models of AML and B-ALL (Toni et al., 2006; Yang et al., 2013; Takam Kamga et al., 2019a,b).

The Notch-dependent role of Wnt/β-catenin was also described in CLL; in this disease, the non-canonical Wnt/PCP/ROR1 is the main activated Wnt signaling and is involved in migration of leukemic cells (Janovska et al., 2016). Constitutive activation of β-catenin is low, but this does not exclude its involvement in the pathogenesis of CLL (Lu et al., 2004; El-Gamal et al., 2014; Mangolini et al., 2018). In fact, CLL cells constitutively express Notch receptors and ligands, whereas MSCs from CLL patients show upregulated Notch receptors and ligands (Kamdje et al., 2012). Culture of primary CLL cells on primary MSCs or EL08-1D2 stromal cell line leads to Notch 2 activation in MSCs, which in turn induces activation of Wnt/β-catenin in co-cultured CLL cells. On the other hand, conditional deletion of Notch2 in MSCs prevents

β-catenin accumulation in CLL cells (Kamdje et al., 2012; Mangolini et al., 2018). Again, the use of Notch inhibitors (GSIs or Notch blocking antibodies) chemosensitizes CLL cells cultured on MSCs monolayer (Kamdje et al., 2012). N-cadherin, a crucial molecule regulating migration and homing of normal hematopoietic cells, is required for the stabilization of β-catenin in co-cultured CLL cells as well as CML cells (Kamdje et al., 2012; Han et al., 2013; Zhang et al., 2013; Mangolini et al., 2018). Consequently, it represents a central mechanism involved in the crosstalk between β-catenin and adhesion molecules to mediate chemoresistance (Toni et al., 2006; Zhang et al., 2013).

In T-ALL, the role of Notch as tumor-driven mechanism has been thoroughly studied, but the influence of stroma-derived Notch signaling is necessary for leukemia cell survival (Ntziachristos et al., 2014) as well as for chemoresistance toward dexamethasone and asparaginase (Iwamoto et al., 2007; Yuan et al., 2013; Cai et al., 2016). Contact with MSCs enhances Notch1, Jagged1, and CD28 expression on T-ALL cells (Yuan et al., 2013) and promotes leukemia cell homing into BM niche in xenotransplantation models; on the other hand, IL-6, SCF, HIF-1α, VEGFα, and Notch ligand Jagged1 is overexpressed in stromal cells (Wang et al., 2016). This aberrant stromal Notch activation negatively regulates CXLC12 in stromal cells, thus hampering their supportive functions toward HSCs and promoting preferentially T-ALL cell development. By contrast, Notch blockade reverts leukemia-associated abnormal blood lineage distribution, thrombocytopenia, and osteoblast functions (Wang et al., 2016). In co-culture, Jagged1 expression on MSCs induces drug resistance in the T-ALL cell line Jurkat, which is prevented by anti-Jagged1 neutralizing antibodies (Yuan et al., 2013). Similarly, the specific β-catenin inhibitor XAV939 may suppress T-ALL cell resistance to cytarabine, thus suggesting that Wnt/Notch cross-talk can be involved in T-ALL and deserves additional investigation (Yang et al., 2013). Overall, the use of Notch or Wnt inhibitors in coculture experiments, impeded increased activity of Notch and Wnt signaling both in leukemia and stromal cells, thus suppress enhancing leukemia survival and drug resistance. The challenge consists in the availability of efficient and safe Notch and Wnt inhibitors.

Pharmacological Strategies to Interfere With Wnt/Notch Signaling in Cancer

Given the importance of Wnt and Notch pathways in cancer development and chemoresistance, numerous pharmacological inhibitors have been developed both as research tools but also as future anticancer drugs (Rizzo et al., 2008; Ntziachristos et al., 2014; Takebe et al., 2015). Inhibitors are designed to target specific steps of the signaling cascade such as ligand-receptors interaction, receptors processing, cytoplasmic effectors, and the formation of transcription complexes (**Table 2**). In the Wnt cascade, inhibitors of ligand-rectors interaction have been developed with regards to natural antagonists of the pathways. Notably, recombinant DKK (DKK-1-4) and SFRP (SFRP1-4) proteins have been developed and used in preclinical experiments to inhibits Wnt signaling in AML, multiple myeloma, and other hematological malignancies (Toni et al., 2006; Chim et al., 2007). Recombinant antibodies directed against Frizzled have also been successfully developed (Pavlovic et al., 2018). Quercetin (a polyphenol) and Niclosamide (an anthelminthic) are both capable to kill leukemia cells and stem cells at least in part by interfering with LRP5/6 (Lu et al., 2011; Maso et al., 2014; Alvarez et al., 2018; Takam Kamga et al., 2020). The post-tranlational addition of porcupine on Wnt ligand is required for the secretion of Wnt proteins. This has served as the basis for the development of Wnt-porcupine inhibitors as WNT974, IWP-2, ETC-159 etc. (Lazzaroni et al., 2016; Kalantary-Charvadeh et al., 2020). Interestingly many inhibitors of this family

TABLE 2 | Notch and Wnt inhibitors.

Inhibitors	Cellular target	References
Secreted Frizzled proteins (sFRPs): sFRP1-5	Ligands (Wnt proteins)	Toni et al., 2006
Dickkopf (DKK) proteins: DKK1-4	Receptors (Frizzled)	Chim et al., 2007
Niclosamide	Co-receptors (LRP5/6)	Lu et al., 2011; Takam Kamga et al., 2020
Quercetin	Wnt antagonist promoters/Co-receptors (LRP5/6)	Maso et al., 2014; Alvarez et al., 2018
WNT974	Porcupine	Lazzaroni et al., 2016
IWP-2	Porcupine	Kalantary-Charvadeh et al., 2020; Takam Kamga et al., 2020
ETC-159	Porcupine	Kalantary-Charvadeh et al., 2020
PKF118-310	β-catenin/TCF/LEF	Leow et al., 2010
PNU-74654	β-catenin/TCF/LEF	Takam Kamga et al., 2020
ICAT	β-catenin/TCF/LEFAPC-Axin interaction	Pongracz et al., 2006
XAV939	Tankyrase	Yang et al., 2013
Anti-Notch1-4, Anti-Jagged1/2	Receptors	Kamdje et al., 2011; Kamdje et al., 2012
Anti-Jagged1/2, Anti-DLL-1/3-4	Ligands	Kamdje et al., 2011; Kamdje et al., 2012
Gamma secretase-I (GSI-I)	Gamma secretase	Baratta, 2019
GSI-IX (DAPT)	Gamma secretase	Grieselhuber et al., 2013; Takam Kamga et al., 2019b
GSI-XII	Gamma secretase	Takam Kamga et al., 2019a
Others GSI-Is	Gamma secretase	Ran et al., 2017; Baratta, 2019
SHAM1	MALM/RBP-jK	Moellering et al., 2009

such as Novartis LGK974 are tested in clinical trials for patients with advanced metastatic solid cancers (Novartis Pharmaceuticals, 2020). Another level of the pathway inhibition is the use of disruptor of the β-catenin/TCF/LEF complexes such as PNU-74654 and PKF118-310 and XAV939. The use of PNU-74654 in association with Ara-C or Idarubicin, abrogate bone marrow protection of AML cells. Similarly, XAV939 suppress T-ALL cell resistance to cytarabine (Leow et al., 2010; Yang et al., 2013; Takam Kamga et al., 2020).

Concerning Notch cascade, ligands, and receptors could be targeted by using Notch receptors/Ligand blocking proteins (Kamdje et al., 2011; Kamdje et al., 2012). Several Notch blocking antibodies are used in clinical trials including OMP-52M51 (anti-Notch1), OMP-21M18 (anti-DLL4), OMP-59R5 (anti Notch2/Notch3) (Andersson and Lendahl, 2014; OncoMed Pharmaceuticals, Inc, 2020). Decoy receptors were also developed to interfere with ligand receptors binding (Funahashi et al., 2008). However, the family of gamma secretase inhibitors (GSIs) has been the main source of the development of Notch inhibitors. They present the unique characteristics to inhibits the activity of all receptors. It is indeed an advantage to exclude redundant activity, but it becomes an inconvenient when only one or two receptors are involved in the cancer process (Ran et al., 2017; Baratta, 2019). Ultimately a transcriptional inhibitor of Notch signaling was synthesized, SAHM1. We provided evidence that SAHM1 could interfere with MSC-induced Notch signaling in AML, abrogating drug resistance (Takam Kamga et al., 2016a).

CONCLUSION

Stromal microenvironment is the major regulator of drug resistance in leukemia, therefore many studies have tried to dissect the molecular mechanisms supporting the pro-survival role of BMME (Agarwal and Bhatia, 2015). The crosstalk between Notch and Wnt signaling acts as a conserved mechanism to promote the BMME-induced chemoresistance of leukemia cells, regardless the leukemia subtype (Sengupta et al., 2007). These pathways can be targeted at different levels of their cascade through several inhibitors, some of them already used in clinical trials, with different results in terms of outcome and toxicity. Thus, Wnt and Notch inhibitors represent potential therapeutic strategies to target leukemia BMME, regardless the underlying molecular signature, thus minimizing the risk of leukemia subclone selection due to the use of inhibitors of specific molecular aberrations (Rizzo et al., 2008; Ntziachristos et al., 2014; Takebe et al., 2015). Most data supporting this view emerge from co-culture studies between leukemia cells and MSCs. Indeed, MSC-based 2D co-culture cannot address cellular heterogeneity and mechanical constrain observed in a 3D BM (Marino et al., 2019). Nevertheless, all the results were successfully translated into different mouse models, thus confirming that *ex vivo* MSC-leukemia cell coculture can be an effective surrogate to investigate BMME interactions *in vitro* and to pave the way toward the identification of new therapeutical approaches capable of overcoming chemoresistance.

AUTHOR CONTRIBUTIONS

PTK designed and wrote the manuscript. RB, GDC, IT, AR, and CT edited the manuscript. AC wrote and edited the manuscript. MK wrote, edited and validated the final version of the manuscript. All authors contributed to the article and approved the submitted version.

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MINI REVIEW
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Endogenous Mobilization of Mesenchymal Stromal Cells: A Pathway for Interorgan Communication?

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To coordinate specialized organs, inter-tissue communication appeared during evolution. Consequently, individual organs communicate their states *via* a vast interorgan communication network (ICN) made up of peptides, proteins, and metabolites that act between organs to coordinate cellular processes under homeostasis and stress. However, the nature of the interorgan signaling could be even more complex and involve mobilization mechanisms of unconventional cells that are still poorly described. Mesenchymal stem/stromal cells (MSCs) virtually reside in all tissues, though the biggest reservoir discovered so far is adipose tissue where they are named adipose stromal cells (ASCs). MSCs are thought to participate in tissue maintenance and repair since the administration of exogenous MSCs is well known to exert beneficial effects under several pathological conditions. However, the role of endogenous MSCs is barely understood. Though largely debated, the presence of circulating endogenous MSCs has been reported in multiple pathophysiological conditions, but the significance of such cell circulation is not known and therapeutically untapped. In this review, we discuss current knowledge on the circulation of native MSCs, and we highlight recent findings describing MSCs as putative key components of the ICN.

Keywords: adipose tissue, native mesenchymal stromal cell, stroma homeostasis, endogenous reservoir, rare cells in circulation

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INTRODUCTION

Each organ is a combination of a functional compartment, the parenchyma, and a stromal compartment, the stroma, supporting the parenchymal cells of the organ (Feeback, 1987). The main function of the stromal compartment is to structure and remodel functional tissue in order to ensure organ homeostasis (Scadden, 2012). In normal tissue, stroma maintains the tissue microenvironment and sustains cell growth in various ways with spatial and temporal self-limitations (Huet et al., 2019). Conversely, stroma imbalance nurtures organ imbalance, which can eventually lead to tumor progression (Valkenburg et al., 2018). Among the cell types residing in the stroma, mesenchymal stem/stromal cells (MSCs) are key components allowing stroma's supportive function. MSCs attract lots of attention because they hold great promise for a multitude

of emerging therapies in regenerative medicine since they promote tissue repair in various degenerative contexts such as osteoarthritis, bone defects, myocardial infarction, inflammatory bowel disease, or neurodegenerative disorders. As such, they have been the subject of clinical trials for more than 20 years (Galipeau and Sensébé, 2018; Pittenger et al., 2019). MSC identification and characterization rely on *in vitro* work, and long steps of culture are needed to collect a usable amount of cells (Dominici et al., 2006). Culture-expanded MSCs consist of a heterogeneous population of cells exhibiting various phenotypes and functional properties, and the extent of these properties depends on the tissue, donor, and species of origin, isolation technique, and culturing protocols (Ankrum et al., 2014). Such variations are known to limit the potential of MSCs for clinical translation, and strategies to enhance engraftment are needed (Hou et al., 2005; Hénon, 2020).

In the past few years, investigating the endogenous repair mechanisms of injured tissues has paved the way for future "*in situ*" strategies to potentiate the body's own repair capacity (Andreas et al., 2014). In this regard, pharmacological activation of endogenous stem cell mobilization from either the blood or a tissue-specific niche is a promising approach (Krankel et al., 2011). Consequently, both triggering and controlling the endogenous mobilization of MSCs represent an additional strategy to achieve effective tissue repair and regeneration. In this review, we present the current state of knowledge and unresolved gaps about the circulation of endogenous MSCs and propose MSC interorgan trafficking as a complementary pathway of communication.

WHAT DO WE KNOW ABOUT THE CIRCULATION OF ENDOGENOUS MESENCHYMAL STEM/STROMAL CELLS?

Circulating Mesenchymal Stem/Stromal Cells: Myth or Reality?

Studies reporting the mobilization, circulation, and recruitment of endogenous MSCs are sparse and heterogeneous (Roufosse et al., 2004) and generated lots of conflicting results (Ojeda-Uribe et al., 1993; Lazarus et al., 1997; Zvaifler et al., 2000; Wexler et al., 2003). Consequently, the presence of blood circulating MSCs is still debated (Mansilla et al., 2006; Wang et al., 2006; Hoogduijn et al., 2014).

Yet, several studies show that endogenous MSCs are found in the bloodstream of various species, but their frequency is rare [0–0.025 colonies/10^{e}6 of peripheral blood mononuclear cells (He et al., 2007)]. Conversely, the circulation of endogenous MSCs greatly increases in response to various types of injuries. Indeed, skeletal traumas, regardless of their severity (Alm et al., 2010), cardiomyopathies (Marketou et al., 2014, 2015), coronary syndrome (Wojakowski et al., 2008), skin burns (Mansilla et al., 2006), liver damages (Chen et al., 2010; Liu et al., 2015), and some types of cancers (Fernandez et al., 1997; Bian et al., 2009) are some examples of clinical situations triggering this increase. Whether endogenous MSCs circulate *in vivo* is not a matter of debate anymore but rather a matter of methods of investigation, time frame (Churchman et al., 2020), and clinical context. Such limitations relate to a lack of precise knowledge of functional, phenotypic, and molecular criteria that define endogenous circulating MSCs.

Immunophenotypic Characteristics of Circulating Endogenous Mesenchymal Stem/Stromal Cells

Despite extensive efforts to characterize MSCs, the definition of *in vivo* identity(ies) of MSCs is still very obscure (Parekkadan and Milwid, 2010). In humans, the canonical MSC surface marker combination $CD13^+/CD44^+/CD73^+/CD90^+/CD105^+/CD34^-/CD31^-/CD45^-$ directly derives from their *in vitro* culture expansion (Dominici et al., 2006). However, many factors, from the harvesting methodology to the conditions of cell culture, dramatically influence MSC phenotype and functions (Bara et al., 2014; Jones and Schäfer, 2015; Pittenger et al., 2019; Walter et al., 2020). In that regard, we and others have demonstrated that cell surface marker profiles of *in vitro* expanded human MSCs differ compared to freshly isolated cells and those residing in their native microenvironment (Sengenès et al., 2005; Maumus et al., 2011; Bara et al., 2014). In particular, the absence of CD34 is considered among the prerequisites to identify MSCs; however, we have shown that CD34 is strongly expressed in native adipose-derived MSCs and that cell culture abolishes its expression (Sengenès et al., 2005; Maumus et al., 2011). Moreover, though some of the MSC markers appear constitutively expressed regardless of environment (Jones et al., 2006), "immunophenotypic drifts" are expected while MSCs circulate. Indeed, the expression of membrane markers such as CD29, CD44, CD73, and CD90, which all regulate MSC adhesion/migration processes, is known to change dramatically to allow MSC detachment and further migration (Rege and Hagood, 2006; Ode et al., 2011; Qian et al., 2012; Xu and Li, 2014). Consequently, using flow cytometry analysis with a combination of surface markers (validated *in vitro*) to detect circulating native MSCs may lead to underestimation and generates conflicting results when compared with studies using functional assays to detect MSCs [such as colony-forming unit-fibroblast (CFU-F) activity] (Fellous et al., 2020; **Figure 1**). Indeed, the level of blood circulating $CD45^-/CD271^+$ MSCs shows higher correlation to CFU-F numbers than the one of $CD45^-/CD73^+/CD90^+/CD105^+$ MSCs (Rebolj et al., 2018). This illustrates that understanding MSC heterogeneity holds promise for refining the definition of MSCs. In that regard, the analysis of MSC heterogeneity from various tissue [bone marrow (BM), adipose tissue (AT), skeletal muscle] is under active investigation using single-cell RNA sequencing technologies (Burl et al., 2018; Hepler et al., 2018; Baryawno et al., 2019; Wolock et al., 2019). However, though powerful, those studies will inform about the signature(s) of native tissue-resident MSC subpopulations but will fail for circulating MSCs. Interestingly, high-throughput technology capable of efficiently capturing

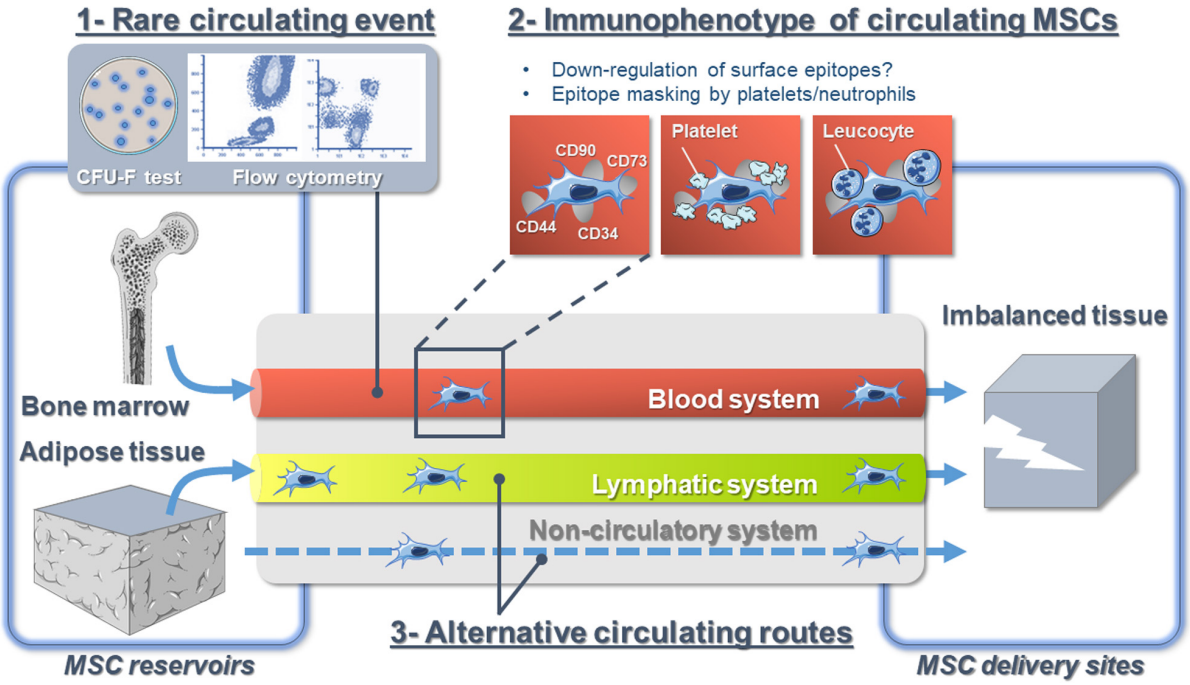

FIGURE 1 | The hypotheses explaining the controversy over endogenous mesenchymal stromal cell circulation *in vivo*.

without marker-based approach and molecularly interrogating rare cells in the circulation at single-cell resolution is under development to study circulating tumor cells (CTCs) (Cheng et al., 2008). Those technologies will be of great utility both to capture and to enable single-cell transcriptome analysis of rare and limited cell populations of circulating endogenous MSCs.

How Do Endogenous Mesenchymal Stem/Stromal Cells Navigate in the Bloodstream?

Little is known about the behavior of MSCs in flowing blood, and our current understanding mostly derives from intravascular infused cultured-expanded MSCs from which we could infer the behavior of native MSCs. While circulating MSCs are always considered to be isolated cells floating in the bloodstream, recent studies demonstrated the close interaction of MSCs with the blood microenvironment. Indeed, using *in vivo* confocal microscopy, it has been reported that the majority of intravascular MSCs are in contact with platelets and/or neutrophils (Teo et al., 2015). Additionally, BM-derived MSCs bind platelets that shield them from surface adhesion, so that they barely adhere at all in the blood flow *via* a mechanism involving podoplanin, the endogenous ligand for C-type lectin-like receptor 2 (CLEC-2) (Sheriff et al., 2018; Ward et al., 2019). CLEC-2 is being expressed broadly, including in platelets, inflammatory leukocytes, and lymphatic endothelial cells. Moreover, platelet depletion decreases MSC trafficking to sites of injury (Langer et al., 2009; Teo et al., 2015). Platelet functions extend beyond the immediate environment of the thrombus (Golebiewska and Poole, 2015). For instance, they play important roles for tissue regeneration (Eisinger et al., 2018), and they also contribute to tumor metastasis (Tesfamariam, 2016). Indeed, it is admitted that CTCs are partly covered with platelets to provide them with "stealth" properties and help their survival in the circulation, where they are challenged by physical forces in the circulation (Nieswandt et al., 1999; Heeke et al., 2019). Whether circulating endogenous MSCs are not single cells traveling the blood alone but are accompanied by other cell types and partners possibly modifying their immunophenotype needs more investigations (**Figure 1**). However, targeting the interaction of MSCs with other cells is a promising tool and future research to improve endogenous MSC detection, collection, and trafficking.

What if Not Just the Blood?

The peripheral blood is considered as the *bona fide* route for native MSC trafficking (He et al., 2007). Indeed, the detection of blood-borne CFU-Fs was earlier (Maximow, 1928) than the detection of BM CFU-Fs (Friedenstein et al., 1968, 1970). However, it is well established that some types of stem cells such as hematopoietic stem cells recirculate daily between the BM and the blood and egress to extramedullary tissues *via* the lymphatic system (Massberg et al., 2007). Until a few years ago, the composition of the lymphatic fluid was virtually unknown. This lack of knowledge was mostly due to the technical difficulty in cannulating lymphatic vessels and the small amount of collected fluid. Over time, some of these technical issues have been resolved, and as such, lymph "omic" composition in physiological and pathological conditions received a lot of

attention (Santambrogio, 2018). However, the precise cellular composition of lymph is still obscure, and immune cell transit was mostly investigated (Platt and Randolph, 2013). Yet, we have demonstrated that MSCs originating from AT, the ASCs (Zuk et al., 2001; Gimble and Guilak, 2003), are found in the lymph fluid in response to lymph node inflammation (Gil-Ortega et al., 2013). Other studies indicate that systemically infused MSCs can be found in secondary lymphoid organs [e.g., mesenteric lymph nodes after intracardiac infusion (Li et al., 2012), lymph nodes, Peyer patches, spleen (Schwarz et al., 2014)]. Interestingly, Han et al. (2020) very recently reported the presence of lots of circulating cells able to form spheroids in the thoracic duct of a mouse model of melanoma, though distant metastases were not developed. Altogether, those data suggest that as cancer cells do, MSCs may exploit several bodily fluid systems as natural transportation routes (Follain et al., 2020; **Figure 1**).

Whether the clinical context, fluid biomechanics, and tissue microenvironment have a role in the initial choice of the fluid route is unknown. As well, accessibility of blood and lymphatic vasculature may strongly influence the pathway taken for MSCs to transit. Finally, flow velocities and shear stress are lower in lymphatic vessels (Dixon et al., 2006), and lymphatic dissemination has been suggested as less deleterious than dissemination through the blood for some type of cancer cells (Wong and Hynes, 2006). Lymph fluid could thus represent a more favorable route for MSCs since their survival may benefit from the passive, low-shear system of fluid transport characteristic of lymphatics. Consequently, an improved understanding of this process might provide a new avenue for targeting MSC transit and might explain conflicting results. At last, the fibroblastic nature of MSCs allows considering extra-circulatory alternative routes, such as connective tissues, for MSC mobilization (**Figure 1**). The potential for such trafficking events, putative mechanisms, and potential functional roles represents important questions for future investigation.

WHICH PHYSIOLOGICAL RESERVOIRS MAY BE MOBILIZED?

MSCs reside in virtually all postnatal organs and tissues; however, not all organs contain the same amount of MSCs (da Silva Meirelles et al., 2006; Crisan et al., 2009). BM is generally considered as the major reservoir of mobilizable MSCs (Koh et al., 2007; Koning et al., 2013). Nevertheless, together with the absence of unique specific markers, the lack of MSC tissue-specific markers impairs the parallel analysis of various physiological reservoirs. Consequently, it is very likely that the role played by extramedullary organs in participating in the pool of circulating endogenous MSCs is underestimated. Indeed, AT is a large source of MSCs, named ASCs (Zuk et al., 2001; Gimble and Guilak, 2003). The uncultured stroma–vascular fraction (SVF) from AT usually contains up to 30% of ASCs. This is 2,500-fold more than the frequency found in BM (Fraser et al., 2008; Baer and Geiger, 2012). Consequently, AT represents so far the largest physiological reservoir of MSCs.

In the attempt of investigating to what extent AT contributes to the pool of circulating endogenous MSCs, we and others have shown that endogenous ASCs are mobilizable and that such mobilization is triggered in response to various types of stresses from inflammation to fat overload (Zhang et al., 2009; Kolonin, 2012; Gil-Ortega et al., 2013, 2014; Girousse et al., 2019). Consequently, AT also largely accounts for the pool of circulating endogenous MSCs, but animal models are still needed to clearly evaluate the respective part played by BM vs. AT.

WHY DO ENDOGENOUS MESENCHYMAL STEM/STROMAL CELLS CIRCULATE?

Whatever the reservoir, circulating endogenous MSCs belong to the group of blood-circulating rare cell populations, classified by Schreier and Triampo (2020) into "constructive" and "destructive" cell types. MSCs are mostly considered as constructive cell types because of their repair and/or homeostasis maintenance properties. The current knowledge on the functional roles of MSCs mainly relies on studies using *in vitro*-expanded MSCs (Keating, 2012; Galipeau and Sensébé, 2018; Pittenger et al., 2019). The struggles in clearly defining native MSCs negatively influence advancement in understanding their role(s) *in vivo* and what is more the role of their circulation. Last, since MSCs virtually reside in all postnatal organs and tissues (da Silva Meirelles et al., 2006; Crisan et al., 2009), one may wonder why MSCs circulate toward distant "injured/inflamed" sites, while resident ones could perform the same activities.

The Interorgan Communication Network

The long-term maintenance of an organism's homeostasis and health relies on the accurate regulation of organ–organ communication (Silverthon et al., 2009). To do so, the central nervous system regulates many organ behaviors using hormones or neurons and organs developed systems to directly communicate their states to one another. This interorgan communication network (ICN) is made up of soluble factors such as peptides, proteins, and metabolites that act between organs to coordinate essential and specialized cellular processes under homeostasis and stress (Droujinine and Perrimon, 2016; **Figure 2**). Recent studies show that more than 15% of the protein-coding genome encodes for roughly 3,000 secreted proteins, but only a handful of them has been properly annotated (Uhlen et al., 2010; Lindskog, 2015). Consequently, the nature of the ICN remains largely a mystery (Droujinine and Perrimon, 2013). The interorgan communication is seen to occur through secreted molecules; however, accumulating data show that organs communicate their state *via* other ways. For instance, extracellular vesicles (EVs) have emerged as a novel messaging system of the organism, mediating cell–cell and interorgan communication (Gould et al., 2003). EVs are secreted membranous structures, entrapping nucleic acids, diverse cellular proteins, and metabolites, and are predicted to transfer their packaged molecules from one cell to another (Gould et al., 2003). EVs traffic to local or distant targets to execute defined biological

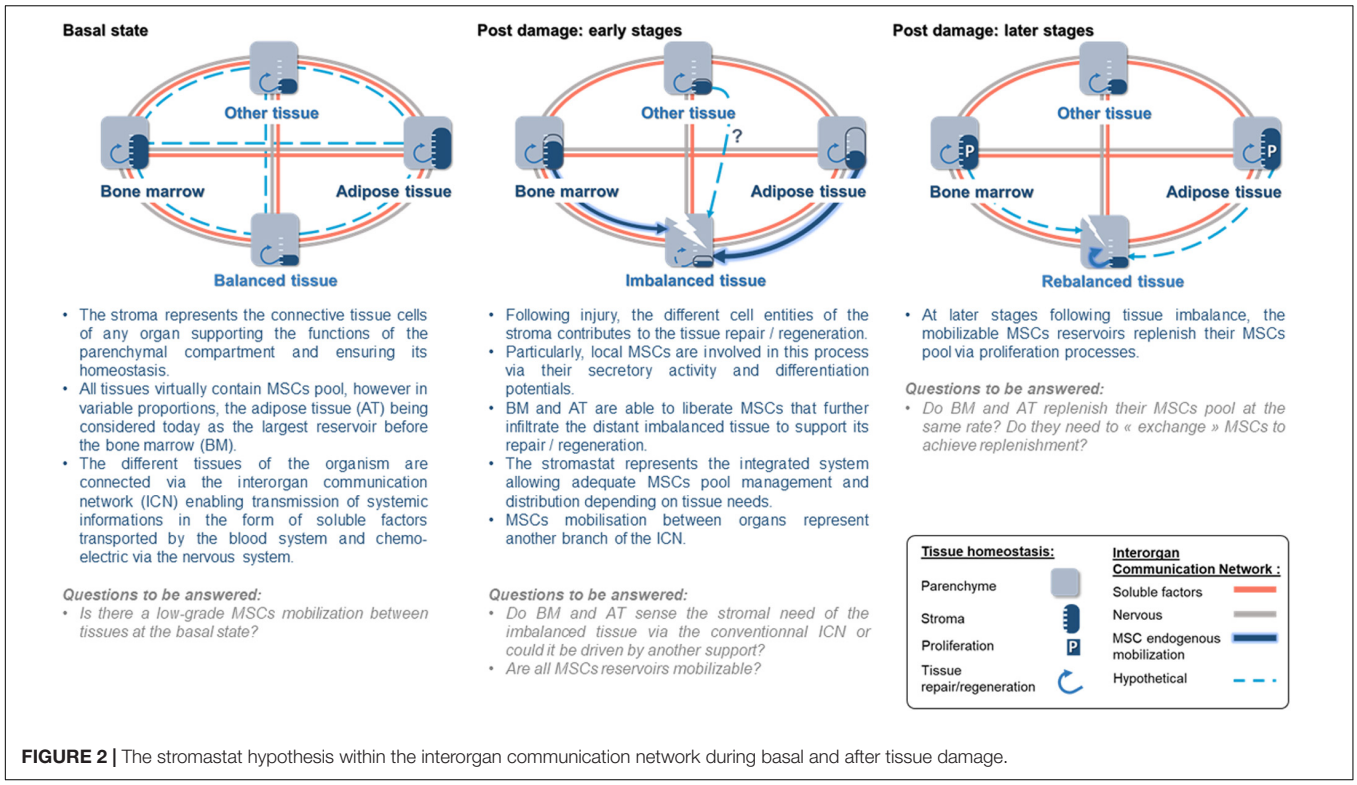

FIGURE 2 | The stromastat hypothesis within the interorgan communication network during basal and after tissue damage.

functions (Théry et al., 2009; Thomou et al., 2017; Margolis and Sadovsky, 2019). Consequently, the ICN encompasses other modes of communication than secreted molecules, and as such, whether the circulation of MSCs is a way of communication between organs needs to be considered.

Circulating Mesenchymal Stem/Stromal Cells, a Way to Communicate Between Bodily Stromas

As stated above, the stromal compartment of each organ structures, nurtures, and remodels the functional compartment to ensure organ homeostasis. MSCs, being a central cellular component of bodily stromas, can be viewed as stroma "sentinel," sensing stromal state and ultimately the organ state. The following scenario regarding the role of circulating endogenous MSCs may be proposed.

Just as suggested for the regulation of body temperature or AT mass in the form of thermostat and adipostat, respectively, bodily stromas could be regulated by a set point to ensure the organism's homeostasis that we will name here the "stromastat." Organ failure leading to stromastat modification could be detected by resident MSCs and trigger the early and rapid transfer from MSC reservoirs to the failing organ. This early transfer of MSCs would support the resident pool of MSCs to allow the rebalance of the failing organ. In parallel, to ensure stromastat, data report that the mobilized reservoir may be replenished (Koning et al., 2013; **Figure 2**). Thus, a possible answer to the meaning of MSC circulation could be that MSCs may represent the cellular part of the ICN. Indeed, evidence that MSCs may belong to the ICN is accumulating. For instance, following organ imbalance (e.g., inflammation, metabolic stress), we observed that ASCs transfer very early from AT toward inflamed lymph nodes (Gil-Ortega et al., 2013) or injured/remodeling skeletal muscle (Girousse et al., 2019). Similar results were also reported for BM-derived MSCs in response to other inflammatory/injury contexts such as myocardial infarction (Fukuda and Fujita, 2005), cranio cerebral trauma (Deng et al., 2011), and encephalomyelitis (Koning et al., 2013). Interestingly, independently of the clinical context or the reservoir investigated, the common point of those studies is the kinetic with which MSCs transfer from their reservoir to the unbalanced site. Such interorgan MSC transfer involves few amounts of cells when compared to the pool of local MSCs. However, we and others have demonstrated that, though discrete, such infiltration dramatically impacts the fate of the organ repair/regeneration/remodeling (Kumar and Ponnazhagan, 2012; Hu et al., 2013; Koning et al., 2013; Girousse et al., 2019). In addition to this, the impact of this rare MSC population could be amplified by the production of EVs, like an inverted funnel effect.

CONCLUSION AND PERSPECTIVES

Both the mobilization and circulation of endogenous MSCs in physiology and pathology are undoubtful as seen in the present review. However, there are still several questions to be resolved before understanding the meaning of such circulation. One can argue that this is merely explainable because of current available technologies and lack of MSC-specific markers. Indeed, being a population of rare cells in the blood, we have only scratched the surface of the potential of circulating MSCs in

diagnostics and regenerative medicine. It appears that in case of "emergency," MSCs traffic from adipose or BM reservoir toward distant "injured/inflamed/imbalanced" organs where infiltrated MSCs trigger local mechanisms to allow repair/regeneration. In return, the MSC provider replenishes its own reservoir so that both compartments balance their respective MSC pools, suggesting the presence of a set point that we suggested to name the "stromastat" (**Figure 2**). How the stromastat regulates organ responses to various stresses and pathological contexts is completely unexplored. This interorgan way of communication may be an unsuspected source of therapeutic targets to help in maintaining whole-organism homeostasis. At last, a better understanding of the control of endogenous MSC circulation, including the description of mobilization and attraction mechanisms, will represent an essential step that will condition their therapeutic potential.

AUTHOR CONTRIBUTIONS

All authors contributed to the design and writing of the manuscript. AG, MM, QS-A, SM, and LC proofread and given comments as well as suggestions. AG designed all the figures. CS supervised and finalized the manuscript.

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Macrophage Control of Incipient Bone Formation in Diabetic Mice

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Both soft and hard tissue wound healing are impaired in diabetes. Diabetes negatively impacts fracture healing, bone regeneration and osseointegration of endosseous implants. The complex physiological changes associated with diabetes often manifest in immunological responses to wounding and repair where macrophages play a prominent role in determining outcomes. We hypothesized that macrophages in diabetes contribute toward impaired osseous wound healing. To test this hypothesis, we compared osseous wound healing in the mouse calvaria defect model using macrophages from C57BL/6J and db/db mice to direct osseous repair in both mouse strains. Initial analyses revealed that db/db mice macrophages showed an inflamed phenotype in its resting state. Incipient bone regeneration evaluated by μCT indicated that bone regeneration was relatively impaired in the db/db mouse calvaria and in the calvaria of C57BL/6J mice supplemented with db/db macrophages. Furthermore, osteogenic differentiation of mouse mesenchymal stem cells was negatively impacted by conditioned medium from db/db mice compared to C57BL/6J mice. Moreover, miR-Seq analysis revealed an altered miRNA composition in db/db macrophages with up regulated pro-inflammatory miRNAs and down regulated anti-inflammatory miRNAs. Overall, this study represents a direct step toward understanding macrophage-mediated regulation of osseous bone regeneration and its impairment in type 2 diabetes mellitus.

Keywords: osteogenesis, db/db mouse, macrophage, bone regeneartion, type 2 diabetes mellitus

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INTRODUCTION

Type 2 diabetes mellitus (T2DM) is a chronic disease of growing prevalence worldwide that has broad and severe systemic consequences (Geiss et al., 2014; IDF Diabetes Atlas, 2020). The impact of altered insulin secretion and insulin resistance on the health of individuals has far-reaching implications that are frequently highlighted by cutaneous ulcers, heart disease, and kidney failure. Elevated inflammation is associated with and, in part, contributory to this pathology (Daryabor et al., 2020). T2DM also influences bone physiology. This is evidenced with respect to bone remodeling and increased risk of bone fractures (Fan et al., 2016), by altered bone density, reduced bone turnover (Reyes-García et al., 2013), and reduced bone repair and regeneration (Follak et al., 2005; Hu et al., 2018). While studies indicate that T2DM influences osteoclastogenesis (Kasahara et al., 2010), hyperglycemia associated with T2DM can influence osteoblastic differentiation (Phimphilai et al., 2017) to affect bone healing (Bhamb et al., 2019). In a human clinical study

using T2DM and body mass index (BMI)-matched control subjects, peripheral blood osteoblastic precursors were significantly reduced in T2DM subjects ($P = 0.0007$) (Sassi et al., 2018). Human osteoblastic cell cultures obtained from hip fracture patients demonstrated that T2DM patients showed marked reduction in *RUNX2* and *OSTERIX* gene expression when exposed to high glucose (Miranda et al., 2016). The impact of diabetes on osteoblast function and the related altered inflammatory signaling remains a central focus of attention.

Several possible mechanisms have been explored concerning how T2DM influences bone physiology, quality, and function. Included are the roles of insulin resistance, enhanced marrow adiposity, formation of advanced glycation end products (AGEs), increased reactive oxygen species, and altered inflammatory factors (Jiao et al., 2015; Chiodini et al., 2020). In T2DM associated with obesity, there is chronic low-grade inflammation and accumulation of pro-inflammatory cells in visceral fat and inflammatory stimulation of tissue macrophages (Wensveen et al., 2015). In fact, the secretion of inflammatory cytokines by multiple cell types is strongly implicated in the inflammatory pathogenesis associated with T2DM (Nikolajczyk et al., 2011). Macrophages are central regulators of inflammation and secrete many of the proinflammatory cytokines and chemokines that direct the inflammatory effects observed in T2DM. They are further required for regulation of wound healing that is well-known to be diminished in T2DM. In cutaneous wounds of diabetic mice, there is increased accumulation of macrophages and sustained accumulation of inflammatory macrophages. The macrophages of diabetic mice are M1-like macrophages, further suggesting an elevated inflammatory phenotype that may impair the regenerative phase of wound healing (Barman and Koh, 2020). The T2DM-associated alteration in macrophage phenotype may also play a role in the regulation of bone repair and regeneration.

Several studies implicate macrophages and their polarization in the regulation of bone regeneration. This may result from the induction of an M2 or wound healing macrophage phenotype (Shi et al., 2018; Wang et al., 2018; Bai et al., 2020). When M2 macrophage-derived extracellular vesicles (EVs) were added to rat calvarial defects within a collagen scaffold, bone healing was increased at 3 and 6 weeks compared to naïve macrophages or M1 EVs (Kang et al., 2020). Given the impact of T2DM on macrophage phenotype and the role that macrophages play in modulating osteogenesis, it is possible that macrophages contribute to the pathophysiology of impaired bone regeneration in T2DM. While several mouse models are available for T2DM-related research including chemical induction of T2DM (King, 2012), the db/db mouse model has been studied with respect to post-natal bone regeneration and serves as a good model for T2DM. Both ectopic osteogenesis and fracture repair have been studied. Delayed mesenchymal osteogenesis and impaired microvascularization were observed (Roszer et al., 2014). In this report, the effect of macrophages on calvarial bone repair was examined in the db/db mouse to investigate the effects of T2DM on bone regeneration.

MATERIALS AND METHODS

Isolation of Bone Marrow-Derived Macrophages

Bone marrow-derived macrophages were isolated from 6-week-old C57BL/6J wild-type (WT) mice (The Jackson Laboratory) and BKS.Cg-Dock7^{m}+/+Lepr^{db}/J db/db (DB) mice (The Jackson Laboratory) as described in Mirza et al. (2013). Briefly, bone marrow cells were flushed out of the mouse femur and tibia and passed through a 40-μm cell strainer. The cells were seeded in 100-mm culture dishes and cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco) containing 20% fetal bovine serum (FBS, Gibco) and 1% antibiotic–antimycotic solution (Gibco). WT macrophages and DB macrophages were obtained by adding 20 ng/ml of recombinant murine M-CSF (PeproTech) into the DMEM growth medium for 7 days. The macrophages were detached by gently pipetting ice-cold phosphate-buffered saline (PBS) containing 5% FBS across the dish followed by incubation at 4°C for 10 min. The cells were then centrifuged at 300 × *g* for 5 min and resuspended in DMEM growth medium for further experiments. For inflammatory stimuli to be induced, macrophages were treated with 100 ng/ml of lipopolysaccharide (LPS) for 24 h.

Flow Cytometry

WT and DB macrophages were preincubated with rat monoclonal [93] TruStain FcX™ anti-CD16/CD32 antibody (101320, BioLegend) to block the Fc receptor prior to further staining. Cell surface antigens were labeled with rat monoclonal [D7] PE/Cyanine7 anti-mouse Ly-6A/E (Sca-1) antibody (108114, BioLegend), rat monoclonal [1A8] Alexa Fluor® 647 anti-mouse Ly-6 antibody (127610, BioLegend), and rat monoclonal [BM8] PE anti-mouse F4/80 antibody (123110, BioLegend). Samples were subjected to analysis using a Gallios flow cytometer and Kaluza software.

Immunocytochemistry

WT and DB macrophages were seeded in 12-well culture dishes and incubated for 18 h at 37°C in 5% CO_2 for immunocytochemistry staining. The coverslips were fixed in 4% paraformaldehyde (PFA) for 15 min, permeabilized using 0.1% Triton X-100 (Fisher Scientific) for 10 min, and blocked with 5% bovine serum albumin (BSA) for 1 h at room temperature. Following incubation with rat monoclonal [CI:A3-1] anti-F4/80 antibody (1/100, ab6640, Abcam), rabbit monoclonal anti-inducible nitric oxide synthase (iNOS) antibody (1/100, ab15323, Abcam), rabbit monoclonal anti-mannose receptor (CD206) antibody (1/100, ab64693, Abcam), mouse monoclonal [3A6] anti-interleukin-1 beta (IL-1β) antibody (1/100, 12242, Cell Signaling), and mouse monoclonal [AC-15] anti-beta actin antibody (1/1,000, NB600-501, Novus Biologicals) overnight at 4°C, cells were then treated with fluorescein isothiocyanate (FITC)- and tetramethylrhodamine (TRITC)-conjugated secondary antibodies (1/1,000, Sigma) for 1 h at room temperature. Cells were imaged using a Zeiss LSM 710 Meta confocal microscope. For quantification of iNOS and IL-1β staining, ImageJ software was used to calculate the fluorescence

intensity. The values were presented as normalized fluorescence intensity divided by the number of nuclei per field ($n = 4$ per group).

qRT PCR

Total RNA was isolated using an RNeasy Mini Kit (Qiagen) as per the manufacturer's protocol. The RNA concentration was measured using NanoDrop One. After first-strand cDNA synthesis was completed, gene-specific primers (**Table 1**) were used to direct PCR amplification and SYBR Green probe incorporation using a Bio-Rad CFX96 thermocycler. All expression data were normalized to housekeeping genes GAPDH, and fold change was calculated using the $^{\Delta\Delta}$Ct method ($n = 4$ per group).

Enzyme-Linked Immunosorbent Assay (ELISA)

Cell culture supernatants were collected from WT and DB macrophages and centrifuged at 1,500 rpm for 10 min. The supernatants were then subjected to an ELISA for detection of cytokine secretion. Mouse IL-6 ELISA kit (Invitrogen) and mouse tumor necrosis factor alpha (TNFα) ELISA kit (Invitrogen) were used per the manufacturer's protocol. The protein concentration of the culture supernatant was measured using a BCA Protein Assay Kit (Thermo Fisher Scientific), and an equal amount of protein was added to each well. The absorbance was measured at 450 nm, and values were presented as concentration in pg/ml according to the standard curve ($n = 3$ per group).

Phagocytosis Assay

Phagocytic activity of WT and DB macrophages were compared using a Vybrant™ Phagocytosis Assay Kit (Invitrogen) as per the manufacturer's recommended protocol. Briefly, WT and DB macrophages were seeded onto 96-well culture dishes (1×10^5 cells per well, $n = 5$ per group). The cells were incubated with fluorescently labeled *Escherichia coli* particles for 2 h and stained with trypan blue. The phagocytosis activity was quantitated by following the internalization of the fluorescent bioparticles, and the relative fluorescence units (RFUs) were measured at 480 nm using a fluorescence plate reader (BioTek plate reader).

Mouse Calvarial Bone Defect Model

Mid-skull transcortical defects were created in 8-week-old mice using a 3.5-mm trephine dental drill. All the defects were filled with collagen scaffolds (BioPlug, BioHorizons). In the experimental groups, scaffolds were populated with 1×10^6 of either WT macrophages (DB+wtMO group) or DB macrophages (WT+dbMO group) per defect. An equivalent volume of PBS was added in the scaffolds of WT and DB groups as a sham-treated control.

After 4 weeks, the mice were sacrificed by carbon dioxide asphyxiation followed by cervical dislocation. The calvariae were harvested, fixed in neutral buffered 4% PFA, and subjected to 3D μCT analysis using a Scanco40 μCT scanner. The μCT scanner data were analyzed using a custom-built Matlab program. All procedures were performed according to animal protocols approved by the Animal Care Committee of the Office of Animal Care and Institutional Biosafety (OACIB) of the University of Illinois at Chicago.

Histology and Immunohistochemistry (IHC)

The calvariae were decalcified in 10% EDTA solution, embedded in paraffin, and sectioned into 5- to 10-μm sections. Hematoxylin and eosin (H&E) staining was performed as per previously published protocols (Huang et al., 2020).

For immunofluorescent staining, the slides were pre-treated with 5% BSA blocking buffer for 1h at room temperature and stained for osteomarkers and macrophage-specific antigens using mouse monoclonal [65529.111] anti-bone morphogenetic protein 2 (BMP2) antibody (1/100, ab6285, Abcam), mouse monoclonal [OCG3] anti-osteocalcin (OCN) antibody(1/100, ab13420, Abcam), rabbit monoclonal anti-iNOS antibody (1/100, ab15323, Abcam), rabbit monoclonal anti-CD206 antibody (1/100, ab64693, Abcam), and mouse monoclonal [3A6] anti-IL-1β antibody (1/100, 12242, Cell Signaling). Sections were then stained with anti-mouse FITC and anti-rabbit TRITC secondary antibodies (1/200, Sigma), imaged using Zeiss LSM 710 laser scanning confocal microscope equipped with Zen image analysis software. For a vascular marker, sections were stained with rabbit polyclonal anti-CD31 antibody (1/100, ab28364, Abcam) and peroxidase-conjugated secondary antibody. ImageJ was used to measure positive immunostained cell number or % area per field ($n = 4$ per group). The positive cell number of iNOS and CD206 was divided by the number of nuclei per field.

Alkaline Phosphatase (ALP) Assay

Human bone marrow-derived mesenchymal stem cells (hMSCs) were purchased from Lonza. hMSCs (5×10^4 cells per well) were seeded in 12-well tissue culture plates and cultured in αMEM (Gibco) containing 20% FBS, 1% antibiotic–antimycotic solution, and 1% L-glutamine (Gibco). Osteogenic differentiation was induced by culturing the cells in αMEM growth medium containing 100 μg/ml of ascorbic acid (Sigma), 10 mM β-glycerophosphate (Sigma), and 10 mM dexamethasone (Sigma) for 5 days.

For preparation of conditioned medium, WT and DB macrophages were seeded into T-25 tissue culture flasks (3×10^6 cells per flask) in DMEM growth medium containing 20 ng/ml of M-SCF overnight. The cells were washed in growth medium and cultured under DMEM−1% FBS condition for 48 h. The culture medium was then harvested and centrifuged at 3,000 × g for 15 min to remove cell debris and added to the hMSCs at an osteogenic medium (OS)-to-CM ratio of 1:1.

hMSCs cultured in osteogenic medium were collected from each well at day 1 and 5. ALP activity was quantified using Alkaline Phosphatase Assay Kit (Abcam) by measuring *p*-nitrophenyl (*p*NP) based on the spectrophotometric absorbance at 405 nm. The fold change of ALP activity at day 5 was calculated with respect to the relative enzymatic activity of day 1.

TABLE 1 | Primer pairs used for qRT PCR.

Genes	Forward (5′-3′)	Reverse (5′-3′)	Size (bp)
GAPDH	AGGTCGGTGTGAACGGATTTG	GGGGTCGTTGATGGCAACA	123
IL-1β	GCAACTGTTCCTGAACTCAACT	ATCTTTTGGGGTCCGTCAACT	89
IL-6	TAGTCCTTCCTACCCCAATTTCC	TTGGTCCTTAGCCACTCCTTC	76
TNFα	CAGGCGGTGCCTATGTCTC	CGATCACCCCGAAGTTCAGTAG	89
IL-10	GCTCTTACTGACTGGCATGAG	CGCAGCTCTAGGAGCATGTG	105
BMP2	GGGACCCGCTGTCTTCTAGT	TCAACTCAAATTCGCTGAGGAC	154
RUNX2	ATGCTTCATTCGCCTCACAAA	GCACTCACTGACTCGGTTGG	146
OSX	ATGGCGTCCTCTCTGCTTG	TGAAAGGTCAGCGTATGGCTT	156

miRNA Sequencing (miR-Seq) Analysis and Quantitative miRNA Expression in Macrophages

RNA isolation from WT and DB macrophages was performed using a miRNeasy Mini Kit (Qiagen) as per the manufacturer's protocol. miR-Seq libraries were constructed using a QIAseq miRNA Library Kit (Qiagen) and sequenced on a NovaSeq 6000 at the UIC Core Genomics Facility. Fastq files were generated with the bclfastq v1.88.4, and adapter sequences and low-quality sequences were removed. miRNAs were identified with BWA ALN.

For qRT PCR, the exact same amounts of miRNA from WT and DB macrophages were utilized to complete cDNA synthesis with a miScript II RT Kit (Qiagen). qRT PCR was performed using a miScript SYBR Green PCR Kit (Qiagen) with custom primer for miR-155-5p: 5′-GGGTTAATGCTAATTGTGATAGGGGT-3′. Relative miRNA expression levels were normalized to RNU6B, and fold change was calculated using the $^{\Delta\Delta}$Ct method ($n = 4$ per group).

Statistical Analysis

For experiments involving two groups, Student's *t*-test with a confidence interval of 95% was utilized. For the experiments involving comparison of more than two groups, one-way ANOVA was performed with a confidence interval of 95%, followed by pairwise comparisons using Tukey's *ad hoc* method ($P < 0.05$).

RESULTS

Resting State of WT and DB Macrophages

We first demonstrated that macrophages isolated from WT and DB mice were similar. Both WT and DB macrophages demonstrate similar surface antigen profiles representative of macrophages as assessed by flow cytometry at the level of Sca-1, Ly6G, and F4/80 (**Figure 1A**). We next analyzed the resting states of WT and DB macrophages at the level of prominent inflammatory mediator expression.

Immunocytochemistry qualitatively affirmed the expression of iNOS and IL-1β only in DB macrophages and of CD206 in both WT and DB macrophages (**Figure 1B**). Furthermore, quantitation of protein expression from immunocytochemical staining in WT and DB macrophages indicated elevated expression of iNOS and IL-1β in the DB macrophages (**Figures 1C,D**). qRT PCR analysis quantitatively demonstrated the differences in the resting inflammatory states of the isolated WT and DB macrophages. Quantitative gene expression analysis of inflammatory cytokines showed that, when compared to WT macrophages, the DB macrophages possess significantly elevated proinflammatory cytokines including a higher expression of IL-1β (**Figure 1E**). ELISA for the other inflammatory cytokines IL-6 and TNFα indicated the reduced expression of IL-6 and an elevated presence of TNFα in the DB macrophages (**Figure 1F**). To observe if the phagocytic ability of the macrophages is altered, a phagocytosis assay was performed. Results presented in **Figure 1G** indicate a small albeit statistically significant reduction in the phagocytic activity of the DB macrophages.

To evaluate if the behavior of WT and DB macrophages is altered when stimulated for an inflammatory response, we treated both types of cells with *E. coli* LPS. Results presented in **Figure 2** demonstrate that DB macrophages show increased presence of IL-1β when subjected to LPS stimulation (**Figures 2A,B**). They also show elevated gene expression levels of TNFα and IL-10 (**Figure 2C**). However, the gene expression levels of IL-1β were less than that of WT macrophage, indicating that the elevated protein expression could be a result of a translationally controlled phenomenon.

Diabetic Macrophages Affect the Quality of Bone Regeneration

Macrophages are capable of modulating osteogenesis (Champagne et al., 2002; Sinder et al., 2015). We proceeded to investigate if incipient bone formation was affected by the DB macrophage phenotype. In the mouse calvarial defect model, we observed by quantitative μCT measurements that the incipient healing at 4 weeks significantly reduced in DB mice compared to WT mice (**Figures 3A,B**). When DB macrophages were introduced into the WT wound sites adherent to collagen scaffolds, they negatively impacted the healing of the WT mouse defects. Conversely, the introduction of WT macrophages into diabetic wounds improved healing. Together with the accompanying histological representation of absence of incipient bone formed in both the DB mouse and WT mouse treated with DB macrophages (**Figure 3A**), these results indicate that DB macrophages impair incipient bone formation. This impairment of osteogenesis in the DB mouse calvaria was partially reversed by the treatment of DB mouse calvarial defects with WT macrophages, further implying a role for the macrophages in the regulation of bone repair.

When the sections were probed with CD31 antibody for the presence of endothelial cells, all the groups showed reduced positive staining in the defect area compared to the WT control

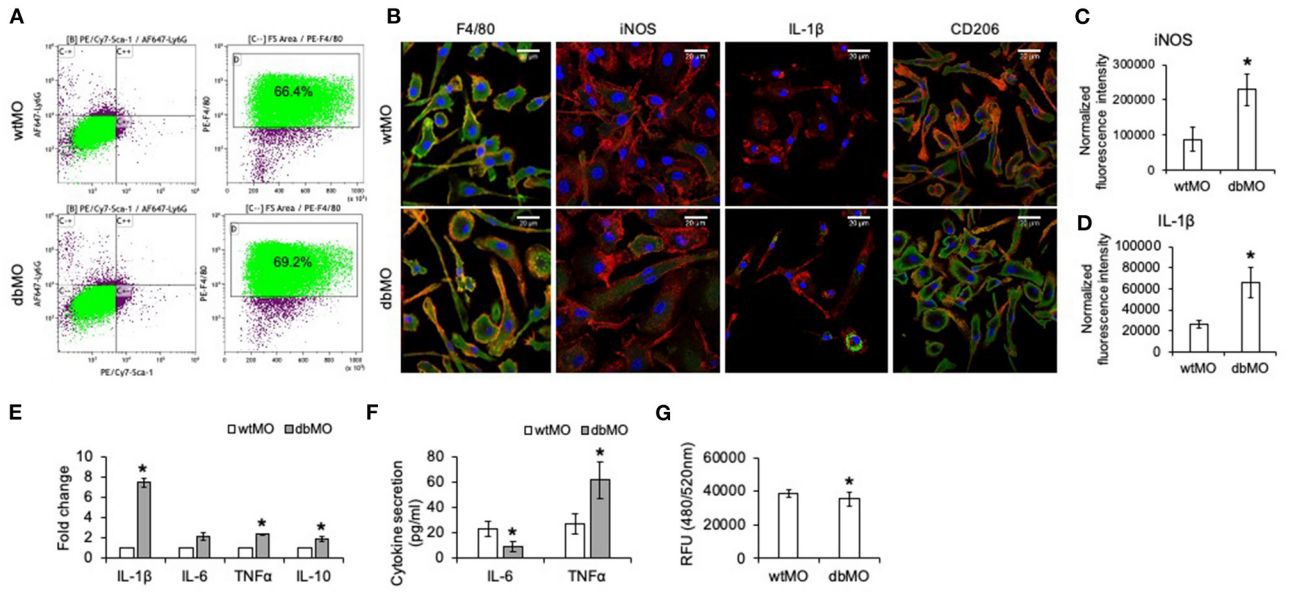

FIGURE 1 | Characterization of WT and DB macrophages. **(A)** Flow cytometric analysis of Sca-1$^-$/Ly6G$^-$/F4/80$^+$ cell population in macrophages isolated from WT and DB mice (wtMO and dbMO, respectively). **(B)** Immunocytochemistry of WT and DB macrophages for F4/80 (green), iNOS (green), CD206 (green), and IL-1β (green). Scale bar represents 20 μm in all images. **(C,D)** Normalized fluorescence intensity of iNOS and IL-1β in immunocytochemistry images using ImageJ software. **(E)** Quantitative RT PCR of WT and DB macrophages for inflammatory cytokines (IL-1β, IL-6, TNFα, and IL-10). The data represent fold change in DB macrophages with respect to WT macrophages ($n = 4$). Note the significantly increased expression of IL-1β in DB macrophages. **(F)** Quantification of IL-6 and TNFα in supernatants from WT and DB macrophages using ELISA ($n = 3$). **(G)** Phagocytosis assay with WT and DB macrophages ($n = 5$). The data represent RFUs. *Statistical significance ($P < 0.05$) calculated by Student's *t*-test.

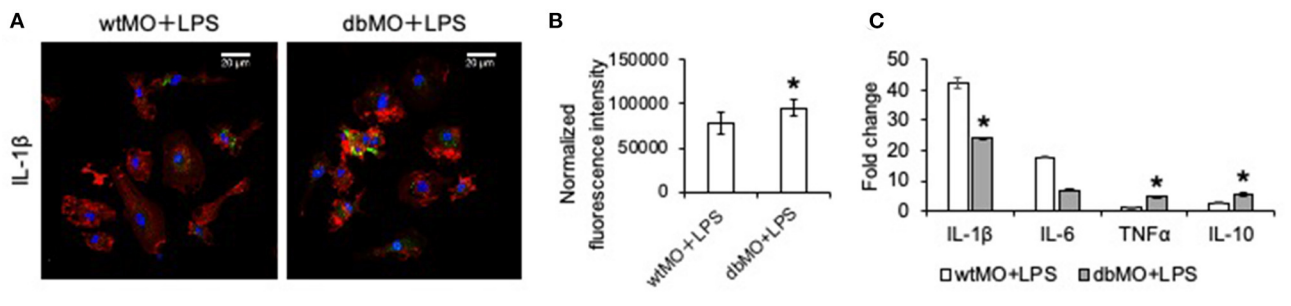

FIGURE 2 | Response of WT and DB macrophages to inflammatory cytokine. **(A)** Representative confocal images of LPS-treated WT and DB macrophages (wtMO and dbMO, respectively), immunostained for IL-1β (green). Scale bar represents 20 μm in all images. **(B)** Normalized fluorescence intensity of IL-1β in immunocytochemistry images ($n = 4$). **(C)** Quantitative RT PCR of WT and DB macrophages for IL-1β, IL-6, TNFα, and IL-10. The data represent fold change in LPS-treated WT and DB macrophages with respect to untreated WT macrophages ($n = 4$). *Statistical significance ($P < 0.05$) calculated by Student's *t*-test.

group (**Figure 3A**). Further, DB mouse calvarial regeneration is associated with reduced vascularization that was not overcome by the addition of WT macrophages in the collagen scaffolds.

To evaluate if the effect of macrophages was mediated by paracrine effects of the macrophage secretome, we performed an *in vitro* mesenchymal stem cell (MSC) differentiation assay in the presence of WT and DB macrophage-conditioned medium (wtCM and dbCM, respectively) and assessed ALP activity. Results presented in **Figure 3C** indicate that ALP activity increased when the MSCs were subjected to an osteogenic differentiation medium with respect to a growth medium. This increase remained unchanged in the presence of wtCM and was significantly reduced (by ∼30%) in the presence of dbCM, indicating the negative effects of the DB macrophage secretome on MSC differentiation.

Further proof of macrophage-associated control of bone regeneration was obtained by IHC evaluation of two key osteogenic protein expressions, namely, BMP2, and OCN (**Figure 4A**). Comparative IHC staining of these proteins in wounds from the four groups of mice demonstrated the relatively low abundance of either protein in the DB mouse defects and WT defects treated with collagen containing DB macrophages

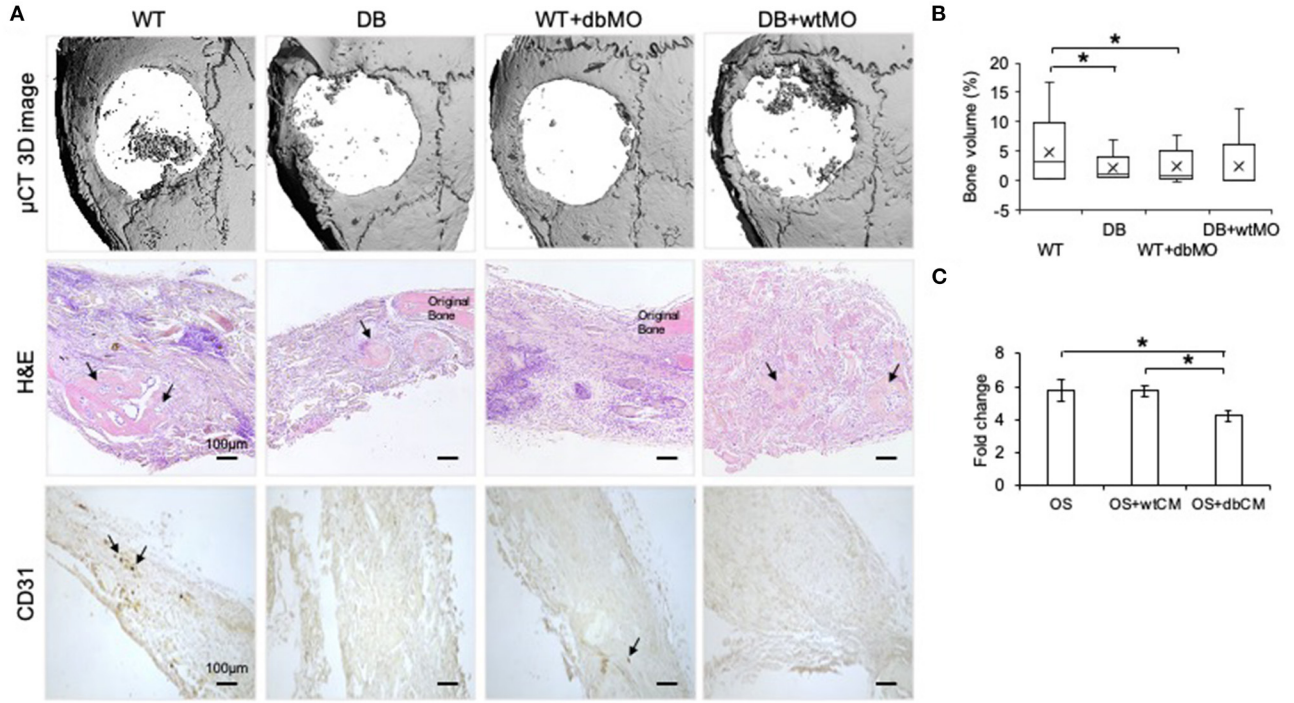

FIGURE 3 | Diabetic macrophages affect the quality of bone regeneration. **(A)** Representative 3D μCT images and light microscopic images of the demineralized and paraffin-embedded tissue sections of the mouse calvaria at 4 weeks post wounding stained with hematoxylin and eosin (H&E) and 3,3′-diaminobenzidine (DAB) for CD31. WT, wild-type mice; DB, db/db mice; WT+dbMO, WT mice treated with DB macrophages; DB+wtMO, DB mice treated with WT macrophages. The black arrows represent newly formed bone in the H&E images and CD31-positive cells in DAB staining images. Scale bar represents 100 μm in all images. **(B)** Volumetric quantitation of the 3D μCT data ($n = 6$). The graph represents mean percentage bone volume regenerated to total volume of the defect ± SD. **(C)** The fold change represents alkaline phosphatase (ALP) activity in hMSCs treated with osteogenic medium (OS), osteogenic medium with WT macrophage conditioned medium (OS+wtCM), and osteogenic medium with conditioned medium from DB macrophages (OS+dbCM) with respect to hMSCs in growth medium at day 5 ($n = 3$). *Statistical significance ($P < 0.05$) as measured by Tukey's *ad hoc* test post ANOVA.

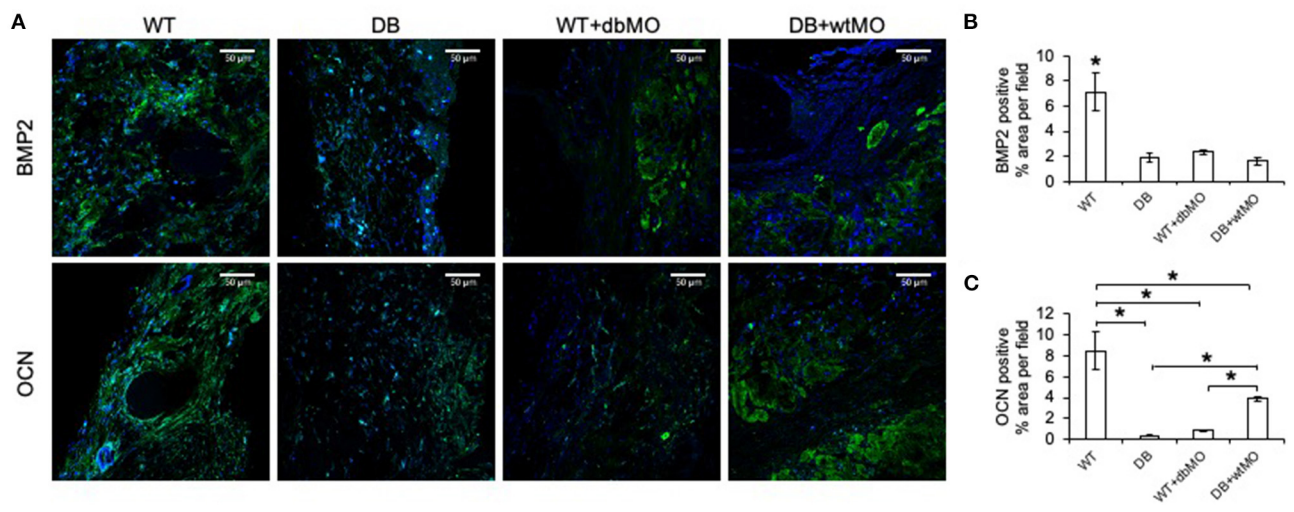

FIGURE 4 | Fluorescent IHC of calvarial sections for BMP2 and OCN. **(A)** Representative confocal micrographs of calvarial sections immunostained for BMP2 (green) and OCN (green). Note the generalized expression of BMP2 and OCN in WT and the relative absence of either protein in DB or WT mouse with the DB macrophages (WT+dbMO) group. Scale bar represents 50 μm in all images. **(B,C)** Quantitation of BMP2- and OCN-positive areas as percentage per field view ($n = 4$). *Statistical significance ($P < 0.05$) as measured by Tukey's *ad hoc* test post ANOVA. WT, wild-type mice; DB, db/db mice; WT+dbMO, WT mice treated with DB macrophages; DB+wtMO, DB mice treated with WT macrophages.

(**Figures 4B,C**). This finding is aligned with the relative absence of incipient bone formation observed in the groups of DB mouse and DB mouse treated with WT macrophages. While BMP2 expression was not significantly improved by treatment of DB mouse defects with WT macrophages, OCN expression levels were significantly improved compared to DB group and the WT group treated with DB macrophages, indicating partial rescue of OCN expression.

Given the relative inflammatory nature of the isolated DB macrophages (**Figures 1**, **2**), we sought to define the inflammatory status of macrophages in healing calvarial defects. **Figure 5A** illustrates the confocal microscopic evaluation of iNOS, IL-1β-positive (M1-like), and CD206 (M2-like) macrophages in healing the calvariae in the four treatment groups. The quantitation of expression of these proteins is represented in **Figures 5B–D**. The iNOS/CD206 (M1/M2) ratio was 4.56-fold in the DB group, whereas it was 0.45-fold in WT group treated with DB macrophages and 0.4-fold in DB group treated with WT macrophages with respect to the WT group. iNOS and IL-1β were present in significantly increased amounts in the DB group compared to the WT group. When WT macrophages were introduced into the DB mouse wounds, the expression levels of both iNOS and IL-1β were significantly reduced, suggesting a reduction in inflammation in these groups. Conversely, when DB macrophages were added to WT defects, the expression levels of iNOS and IL-1β were increased. While the iNOS expression increase was not statistically significant, a significant increase in IL-1β expression was observed. With respect to CD206, a marker for M2-like cells, DB mice displayed a reduced presence of this marker, and its expression increased with the addition of WT macrophages to DB wounds. Interestingly, the addition of DB macrophages to WT wounds dramatically increased the expression levels of this marker. Relatedly, the relative absence of CD206 staining in DB mouse calvarial defects suggests a reduced level of reparative macrophages that is associated with the absence of incipient bone repair in this group However, the treatment of either DB mouse calvarial defects with WT macrophages or WT defects with DB macrophages was associated with an increased number of CD206-positive cells at the defect sites of both types of animals. The introduction of WT macrophages to the diabetic wounds shifted this ratio toward normalcy and was associated with improved incipient bone repair. The diabetic bone defects showed impaired early healing that was associated with the presence of the inflammatory state of the macrophages and the altered ratio of inflammatory to reparative macrophages.

Possible Role of miRNA in the Function of WT and DB Macrophages

Our results indicated that the resting states of the WT and DB macrophages are varied and that their response to pro-inflammatory stimulus is different. To evaluate the possible role of miRNAs in WT and DB macrophages, we performed miR-Seq analysis of the two isolated cell types. The table presented in **Figure 6A** lists the top 25 most abundant miRNAs in DB macrophages and their expression levels in WT macrophages. **Figure 6B** lists the top 25 miRNAs in WT macrophages and their corresponding expression in DB macrophages. The green color coding denotes pro-inflammatory miRNA showing increased expression in DB macrophages compared to WT, and the red color coding denotes anti-inflammatory miRNA showing reduced expression in DB macrophages compared to WT. In addition to these miRNAs, we also evaluated the expression of miR-155-5p in WT and DB macrophages by qRT PCR and its change in the presence of LPS treatment. miR-155 is characterized as a master regulator of inflammation (Mahesh and Biswas, 2019), and we have demonstrated the role of exosomal miR-155 in bone repair. miR-155-5p was downregulated in resting DB macrophages (**Figure 6C**). However, upon LPS stimulation, DB macrophages showed a more robust increase in miR-155-5p compared to WT macrophages, indicating their propensity for an enhanced inflammatory response to stimuli.

DISCUSSION

T2DM is a disease of epidemic proportion affecting ∼10.5% of the US population (34.2 million people in 2020) (Centers for Disease Control and Prevention, 2020). It is characterized by impaired insulin secretion, glucose intolerance, and hyperglycemia (American Diabetes Association, 2010). As a result, multiple organ systems are affected and cause significant comorbidities. A common underlying sequela of T2DM is immune dysregulation and inflammation. In addition to changes in the T-cell compartment and reduction in regulatory T cells and NK cells, there is reported abnormal polarization of macrophages (Zhou et al., 2018). The alterations in immune cell function in T2DM may have subsequent effects on other tissues and generally manifest as challenges in wound healing (Barman and Koh, 2020). The DB macrophage displays a pro-inflammatory phenotype as shown above and suggests that this diabetes-related macrophage phenotype is involved in the modulation of bone repair. This may explain, in part, how engraftment of the DB macrophage impaired bone regeneration in WT mice.

This finding of reduced bone regeneration in DB mice and the finding that DB macrophages impair bone regeneration in WT mice are consistent with the existing evidence that bone healing is impaired in T2DM. A 2015 review indicated the increased risk of fracture in T2DM and suggests that the impairment of bone formation is associated with increased osteoblast apoptosis and reduced expression of osteoinductive factors (Marin et al., 2018). A more recent review has also highlighted the effect of T2DM on MSC differentiation leading to alterations in vascularization and increased number of adipocytes, which negatively impact bone regeneration (Cassidy et al., 2020).

The number of bone marrow MSCs is reduced in T2DM (Cassidy et al., 2020), suggesting a systemic role for heightened inflammation in bone regeneration. A differential gene expression study of blood from T2DM and non-diabetic patients revealed low bone morphogenetic protein 4 (*BMP4*), *BMP7*, and *RUNX2* expression in T2DM patients. Further, metformin was observed to enhance *BMP4* levels and osteogenic function of MSCs (Liang et al., 2020). Impaired bone repair in

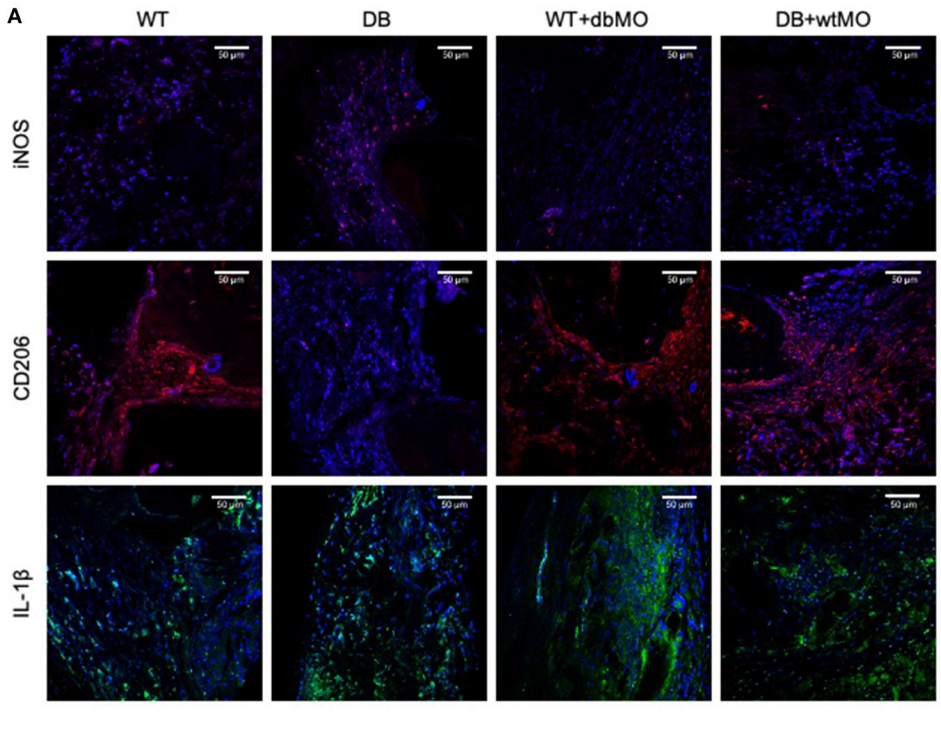

FIGURE 5 | Fluorescent IHC of calvarial sections for iNOS, CD206 and IL-1β. **(A)** Representative confocal micrographs of 4-week calvarial sections stained for iNOS (red), CD206 (red), and IL-1β (green). **(B,C)** Quantitation of iNOS- and CD206-positive cells as percentage per field view ($n = 4$). Note the increase of iNOS-positive cells and the absence of CD206-positive cells in the DB group and the increase in the number of CD206-positive cells in the DB with WT macrophages (DB+wtMO) group compared to the DB group. **(D)** Quantitation of the IL-1β-positive area as percentage per field view ($n = 4$). In all images, nuclei are stained with DAPI. Scale bar represents 50 μm in all images. *Statistical significance ($P < 0.05$) calculated by Tukey's *ad hoc* test post ANOVA. WT, wild-type mice; DB, db/db mice; WT+dbMO, WT mice treated with DB macrophages; DB+wtMO, DB mice treated with WT macrophages.

diabetics, demonstrated in this model of bone repair in T2DM, may be influenced by reduced numbers or activity of MSCs in osteogenesis.

The db/db mouse model is a recognized model of T2DM. Previous studies of bone regeneration conducted in this model indicate that db/db mice display reduced bone repair (Wallner et al., 2015). For example, using a 1-mm unicortical defect, absence of bone regeneration was recovered by treating defects with syngeneic adipogenic stem cells (Wallner et al., 2016). While suggesting that bone regeneration was recovered by augmenting the number of osteoinductive stem cells, the tibia defects are not critical-sized defects, and potential paracrine effects of the implanted cells were not considered. Obesity does complicate the interpretation of studies with db/db mice. Obesity-related inflammation, however, does increase the pro-inflammatory phenotype of macrophages. While obesity is a comorbidity of diabetes, we did not intend for this work to explain obesity-mediated effects on macrophage function in bone repair. To separate the effects of obesity from T2DM, further studies need to be performed wherein the effects observed in db/db mice are directly compared to those in non-diabetic obese mice. In this study, we isolated macrophages from DB mice and placed them in WT wounds. These WT animals were not obese and yet displayed altered healing when engrafted with DB macrophages. Based on these observations, we demonstrated that the macrophage of the DB mouse altered bone repair associated with their altered inflammatory status (M1/M2 ratios).

T2DM-related impairment of osteogenesis has been previously observed in the Zucker diabetic fatty (ZDF) rat calvarial model in which partial bone regeneration was observed at 8 weeks, but significantly reduced amounts of bone with reduced angiogenesis were found in critical-sized defects of the diabetic rat calvaria (Caliaperoumal et al., 2018). Separate studies in the ZDF rat indicate that osteoblastogenesis is suppressed in T2DM (Hamann et al., 2011). Not only are osteoblasts affected, osteoclastogenesis and osteoclast activity is also reduced in a rat model of T2DM (Hu et al., 2019). The early time point investigated in this study did not warrant a study of osteoclastogenesis. However, this serves as a limitation of this study, and further studies are required to ascertain the role of diabetic macrophages in osteoclastogenesis and bone resorption.

The macrophage is a known significant factor in T2DM pathophysiology. The monocyte/macrophage population contributes to chronic inflammation that impairs wound healing

A

Sequence	Number of Copies		
	DB macrophage	WT macrophage	DB/WT
mmu-let-7f-5p	239083.929	66709.58861	3.583951483
mmu-miR-21a-5p	196664.9278	30974.71503	6.349208624
mmu-miR-16-5p	195062.4482	50642.65328	3.851742268
mmu-miR-142a-3p	162927.3144	50966.24498	3.196769047
mmu-let-7a-5p	114956.9339	31774.62236	3.617885135
mmu-miR-29a-3p	106418.1154	25926.6214	4.104588631
mmu-let-7i-5p	102569.829	102908.156	0.99671234
mmu-miR-27b-3p	80009.92496	24798.70474	3.226375159
mmu-miR-342-3p	36210.30923	2815.926214	12.85911153
mmu-miR-221-3p	33710.86468	17804.4855	1.893391678
mmu-miR-34a-5p	27719.01392	7213.996537	3.842393571
mmu-let-7c-5p	25302.39537	17445.86828	1.450337408
mmu-miR-26a-5p	24744.60972	6532.4187	3.78797056
mmu-miR-29b-3p	24253.35644	3571.34165	6.791105085
mmu-miR-29c-3p	19923.3213	2716.844991	7.333256541
mmu-miR-191-5p	17143.35456	893.7820484	19.18068794
mmu-let-7j	15970.89129	3851.3881	4.146788348
mmu-miR-23a-3p	14491.2929	3460.427479	4.187717553
mmu-let-7g-5p	14350.21712	4216.000688	3.403751133
mmu-miR-30d-5p	12098.70747	2329.670914	5.19331181
mmu-miR-24-3p	11736.71708	1658.506071	7.0766802
mmu-miR-103-3p	11715.39956	4198.172379	2.790595169
mmu-miR-125b-5p	11265.69507	3423.824225	3.290383596
mmu-let-7b-5p	10313.19596	7270.479145	1.418502928
mmu-miR-146a-5p	8993.139463	49882.34689	0.180287018

[Top 25 most abundant miRNAs in DB macrophages]

B

Sequence	Number of Copies		
	DB macrophage	WT macrophage	DB/WT
mmu-let-7i-5p	102569.829	102908.156	0.99671234
mmu-let-7f-5p	239083.929	66709.58861	3.583951483
mmu-miR-142a-3p	162927.3144	50966.24498	3.196769047
mmu-miR-16-5p	195062.4482	50642.65328	3.851742268
mmu-miR-146a-5p	8993.139463	49882.34689	0.180287018
mmu-let-7a-5p	114956.9339	31774.62236	3.617885135
mmu-miR-21a-5p	196664.9278	30974.71503	6.349208624
mmu-miR-29a-3p	106418.1154	25926.6214	4.104588631
mmu-miR-27b-3p	80009.92496	24798.70474	3.226375159
mmu-miR-221-3p	33710.86468	17804.4855	1.893391678
mmu-let-7c-5p	25302.39537	17445.86828	1.450337408
mmu-miR-223-3p	4298.534101	8689.64411	0.494673206
mmu-let-7b-5p	10313.19596	7270.479145	1.418502928
mmu-miR-34a-5p	27719.01392	7213.996537	3.842393571
mmu-miR-26a-5p	24744.60972	6532.4187	3.78797056
mmu-miR-22-3p	8415.258323	6447.85256	1.305125737
mmu-miR-27a-3p	8285.588095	4279.583065	1.936073671
mmu-let-7g-5p	14350.21712	4216.000688	3.403751133
mmu-miR-103-3p	11715.39956	4198.172379	2.790595169
mmu-miR-378a-3p	1723.459895	4063.434538	0.424138713
mmu-let-7j	15970.89129	3851.3881	4.146788348
mmu-let-7d-5p	7988.636485	3850.441464	2.074732614
mmu-miR-29b-3p	24253.35644	3571.34165	6.791105085
mmu-miR-23a-3p	14491.2929	3460.427479	4.187717553
mmu-miR-125b-5p	11265.69507	3423.824225	3.290383596

[Top 25 most abundant miRNAs in WT macrophages]

C

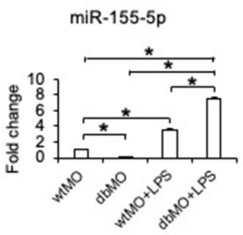

FIGURE 6 | miR-Seq analysis of WT and DB macrophages. **(A)** A list of the top 25 most abundant miRNAs in DB macrophages and their expression levels in WT macrophages. **(B)** Top 25 most abundant miRNAs in WT macrophages and their expression levels in DB macrophages. The pro-inflammatory miRNAs are highlighted in green, and anti-inflammatory miRNAs are highlighted in red. Note that the increased expression of pro-inflammatory miRNAs (miR-142a-3p, miR-342-3p, and miR-191-5p) and suppressed expression of anti-inflammatory miRNAs (miR-146a-5p, miR-223-3p, and miR-378a-3p) in DB macrophages compared to WT. **(C)** Quantitative RT PCR of WT macrophages (wtMO) and DB macrophages (dbMO) for miR-155-5p. The graph showing the expression level of miR-155-5p in WT and DB macrophages with or without LPS stimulation. Data represent mean fold change with respect to WT macrophage expression ± SD ($n = 4$). Note the significant increase of miR-155-5p expression upon LPS treatment in DB macrophages (dbMO+LPS). *Statistical significance ($P < 0.05$) calculated by Tukey's *ad hoc* test post ANOVA.

and may reflect a greater number of macrophages and their dysregulation (Barman and Koh, 2020). Studies that deplete monocytes/macrophages from mice (clodronate treatment or MaFIA mice) demonstrate that the absence of macrophages impairs bone regeneration (Davison et al., 2014; Kaur et al., 2017). Based on the concept that macrophages are one of the primary regulators of wound healing, the present investigation involved the syngeneic transplantation of DB macrophages in WT wound that resulted in impaired osteogenesis. The larger number of macrophages and their relatively pro-inflammatory phenotype contributed to the reduction in bone repair in the WT mice when DB macrophages were implanted in them. Conversely, transplantation of WT macrophages into the DB mouse partially reconstituted the impaired regenerative process in these diabetic osseous wounds. We speculate that this partial restoration may be attributed to the reduction in the inflammatory status of the wound bed, resulting in altered M1/M2 ratios with an increase in the M2 macrophage population favoring bone repair.

While the results of this study are solely focused on the influence of diabetic macrophages (as indicated here by increased numbers of CD206-positive cells) in bone repair, as discussed previously, other effects of T2DM should also be considered. For example, the role of insulin resistance requires further investigation. A recent study identified that insulin resistance as a result of T2DM negatively affects bone regeneration (Srikanthan et al., 2014). On the other hand, the anabolic role of insulin in bone formation has also been documented and reviewed (Thrailkill et al., 2005). This dichotomy is currently debated at molecular and mechanistic levels, and it would be interesting to investigate the role of macrophages in this process. We envision that the results presented here outlining the role of diabetic macrophages may serve as a starting point for such investigations.

In this large, critical-sized defect, islands of osteogenesis formed remotely from the bone surfaces populated by bone-specific macrophages termed osteomacs (Miron and Bosshardt, 2016; Batoon et al., 2017) are likely less influenced by these regulatory macrophages that form canopy structures above progenitor cells lining the bone surface. Circulating cells also contribute to wound healing, and studies in the parabiotic mouse model demonstrate the contribution of circulating cells to osteogenesis in fracture repair (Kumagai et al., 2008), indicating that large defects are populated by cells derived from the circulation. Here, as in other wounds, macrophages are likely derived from circulating monocytes (Rodero et al., 2014).

The implanted macrophages may exert their effects on osteoprogenitors in the local environment by both direct cell–cell and soluble factor signaling (Champagne et al., 2002; Pajarinen et al., 2019). ALP activity was reduced in differentiating MSCs

in the presence of dbCM when compared to culture in the presence of wtCM. The measured differences in BMP2 and OCN expression further imply that the observed absence of incipient bone formation in the DB mice or in WT mice treated with DB macrophages is due, in part, to paracrine effects of the DB macrophage upon the osteoprogenitor cells. Factors secreted by macrophages and known to reduce OSTERIX and BMP2 expression include IL-1β, IL-6, and TNFα (Nakase et al., 1997). Of note, all these pro-inflammatory factors' gene expression was elevated in the DB macrophages relative to the WT macrophages, again suggesting that the macrophage phenotype of the DB mouse directly impairs osteoblastogenesis.

In addition to growth factors, cytokines, and chemokines, cells secrete exosomes [30- to 150-nm extracellular vesicles containing protein and miRNA cargo (Golchin et al., 2018)] that transfer their cargo as regulatory signals from parental to target cells. Exosomes serve as a carrier for miRNA from parental cells to target cells, and exosomal miRNAs are implicated as the primary effectors of exosome function (Huang et al., 2020). In a recent study, we have highlighted the role of macrophage-derived exosomal miRNA in bone repair (Kang et al., 2020). In this manuscript, we have performed a miR-Seq analysis of the miRNA composition of macrophages from WT and DB mice. Of the top 25 most abundantly expressed miRNAs in the WT and DB macrophages, we have identified miRNA candidates that have proven roles in inflammation. Notably, our results indicate the upregulation of pro-inflammatory miRNAs (Wei et al., 2013; Gu et al., 2017; Mandolesi et al., 2017) and the downregulation of anti-inflammatory miRNAs in DB macrophages (Rückerl et al., 2012; Testa et al., 2017; Cheng et al., 2020; Kang et al., 2020), indicating that the transfer of miRNA via paracrine mechanisms may also play a role in macrophage-mediated control of bone repair. In addition to the miRNA presented in the table, we have also evaluated the changes in expression levels of miR-155. miR-155 is considered a master regulator of inflammation affecting the NLRP3 inflammasome pathway (Mahesh and Biswas, 2019). The stimulated macrophage expression of miR-155 in DB macrophages is greater when compared to WT macrophages, further indicating the sensitized pro-inflammatory state of the macrophage phenotype in DB mice. Further studies on the role of diabetic macrophage miRNAs and the paracrine role of these miRNAs via exosomes in relation to bone repair and in general tissue repair are warranted to understand this complex process in greater detail.

CONCLUSION

The role of macrophages in the pathology of bone healing observed in diabetes was modeled in the DB mouse. Transfer of DB macrophages to WT mouse calvarial defects impaired incipient bone repair *in vivo* and affected M1/M2 ratios in the wound bed, and the macrophage-derived conditioned medium impaired ALP activity in differentiating MSCs *in vitro*, suggesting that the paracrine effects of macrophages on incipient osteogenesis may impair bone formation in the DB mouse. The partial restoration of incipient bone formation in the DB mouse by transfer of WT mouse macrophages to calvarial wounds further suggests that macrophages play an important role in the impairment of osteogenesis in diabetes. Targeting the inflammatory phenotype of the diabetic macrophage may provide an alternative therapeutic strategy to enhance bone repair in diabetic patients.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: BioProject ID PRJNA685165.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Care Committee of the Office of Animal Care and Institutional Biosafety (OACIB) of the University of Illinois at Chicago (protocol number 20-020).

AUTHOR CONTRIBUTIONS

LC and MK conceived of the project and experimental design. MK, GT, C-CH, YL and SS contributed to the laboratory and animal experiments. LC, SR, and MK prepared this manuscript. All authors contributed to the article and approved the submitted version.

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frontiers in Cell and Developmental Biology

REVIEW
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Eph-Ephrin Signaling Mediates Cross-Talk Within the Bone Microenvironment

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Skeletal integrity is maintained through the tightly regulated bone remodeling process that occurs continuously throughout postnatal life to replace old bone and to repair skeletal damage. This is maintained primarily through complex interactions between bone resorbing osteoclasts and bone forming osteoblasts. Other elements within the bone microenvironment, including stromal, osteogenic, hematopoietic, endothelial and neural cells, also contribute to maintaining skeletal integrity. Disruption of the dynamic interactions between these diverse cellular systems can lead to poor bone health and an increased susceptibility to skeletal diseases including osteopenia, osteoporosis, osteoarthritis, osteomalacia, and major fractures. Recent reports have implicated a direct role for the Eph tyrosine kinase receptors and their ephrin ligands during bone development, homeostasis and skeletal repair. These membrane-bound molecules mediate contact-dependent signaling through both the Eph receptors, termed *forward signaling*, and through the ephrin ligands, referred to as *reverse signaling*. This review will focus on Eph/ ephrin cross-talk as mediators of hematopoietic and stromal cell communication, and how these interactions contribute to blood/ bone marrow function and skeletal integrity during normal steady state or pathological conditions.

Keywords: bone marrow microenvironment, bone marrow mesenchymal stem cells, osteogenic differentiation, hematopoietic stem cells, osteoimmunology, vasculature, musculoskeletal pathology, Eph-ephrin communication

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INTRODUCTION - THE CELLULAR COMPONENTS OF BONE MICROENVIRONMENT

The bone microenvironment provides cellular, molecular, and metabolic stimuli in an endocrine, paracrine and autocrine manner to regulate and maintain skeletal integrity, support hematopoiesis and regulate immune cell responses. The cellular components that reside within the bone microenvironment include endothelial cells, perivascular cells, neural cells, Schwann cells, and those of the mesenchymal and hematopoietic lineages. These populations contribute to specific stem cells niches located within the bone marrow and the bone to support and maintain hematopoiesis and osteogenesis (**Figure 1**) (Chan et al., 2015; Ramasamy et al., 2016; Crane et al., 2017). Hematopoiesis is sustained by hematopoietic stem cells (HSC) that give rise to the erythroid (erythrocytes, megakaryocytes, platelets), myeloid (basophil/ mast cells, eosinophils, neutrophils, dendritic cells, monocytes, macrophages and osteoclasts) and lymphoid (T-lymphocytes, B-lymphocytes and natural killer cells) lineages. Mesenchymal stem cells (MSC)

give rise to cells of the chondrogenic lineage (chondroprogenitors, proliferating, resting, pre-hypertrophic and hypertrophic chondrocytes), osteogenic lineage (osteoprogenitors, osteoblasts, bone lining cells, osteocytes), stromal cells, reticular cells, smooth muscle cells and adipocytes (**Figure 1**). Maintenance of the bone microenvironment under physiological or pathological conditions is dependent on interactions between the different cellular components, as well as their precise anatomical location within the skeleton. However, a better understanding of the numerous molecular interactions that mediate intercellular signaling and function within the bone microenvironment is required to help identify novel therapeutic strategies to treat musculoskeletal conditions. The present review describes the erythropoietin-producing human hepatocellular (Eph) receptor tyrosine kinase family and the Eph receptor interacting protein (ephrin) ligands (also termed Efn molecules) that are expressed by stromal, hematopoietic, and vascular populations and the function of Eph/ephrin molecules within the bone microenvironment.

OVERVIEW OF THE EPH-EPHRIN MOLECULES

The Eph family of receptor tyrosine kinases (RTKs) and their ephrin ligands are contact-dependent, cell membrane-bound molecules expressed by invertebrates and vertebrate species. This family consists of two subclasses, the A subclass and the B subclass, comprising 14 Eph receptors (EphA1-8 and EphA10 and EphB1-4 and EphB6) and eight ephrin ligands (ephrin-A1-A5 and ephrin-B1-B3) in humans (Kania and Klein, 2016; Nguyen et al., 2016a; Liang et al., 2019). The Eph-ephrin intercellular and intracellular signaling modalities, both catalytic and non-catalytic, are complex. The structure of these molecules, the size of the family, and the range of activation-dependent downstream effects all contribute to this complexity (Gale et al., 1996; Kania and Klein, 2016; Nguyen et al., 2016a; Liang et al., 2019).

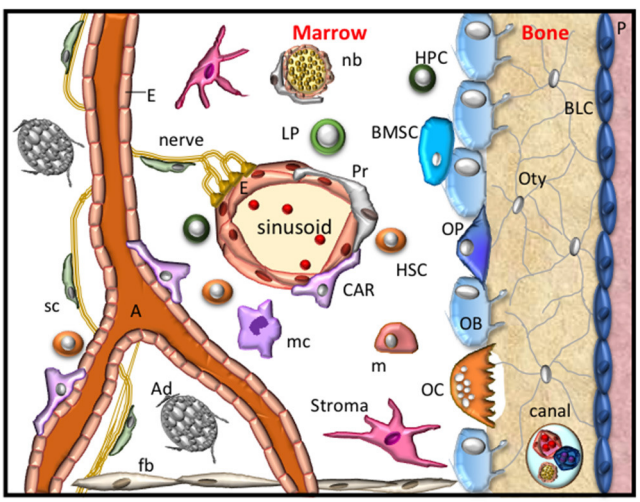

FIGURE 1 | The bone microenvironment. A schematic representation of the resident cells within the bone microenvironment during homeostasis. These cellular components include fibroblasts (fb), stroma, bone marrow stem cells (BMSC), osteoprogenitors (OP), obsteoblasts (OB), bone lining cells (BLC), osteocytes (Oty), the periosteum (P), nerves, Schwann cells (sc), nerve bundle (nb), arterioles (A), endothelial cells (E), Pericytes (Pr), CXCL12-abundant reticular cells (CAR), also known as Leptin Receptor+ mesenchymal stromal cells, adipocytes (Ad), hematopoietic stem cells (HSC), hematopoietic progenitor (HPC) cells, lymphoid progenitors (LP), monocytes (m), macrophage (mc) and osteoclasts (OC). These cells form specific niches to regulate haematopoiesis and osteogenesis and thus maintain skeletal integrity.

It is important to note that there is promiscuous binding within subclasses, where multiple EphA receptors bind with differing affinity to cognate ephrin-A ligands and EphB receptors bind with ephrin-B ligands. The Eph receptors of both subclasses are predominantly structurally conserved, with the extracellular region consisting of the globular ligand binding domain, a cysteine-rich region, encompassing the Sushi and epidermal growth factor like domains, and two fibronectin III repeats. Intracellularly, Eph receptors consist of a juxtamembrane, a kinase domain, a sterile alpha motif (SAM) domain and a postsynaptic density, discs-large, zona occludens-1 (PDZ) domain. The most variation within these receptors lies in the ligand binding domain and thus the receptors are functionally divided into two subclasses determined by the binding affinity for their cognate ligand (Gale et al., 1996), with minimal interaction between subclass, with the exception of EphA4 which can bind with ephrin-B ligands; and EphB2 which can also interact with ephrin-A5 (Gale et al., 1996; Holland et al., 1998; Himanen et al., 2004).

Conversely, the ephrin ligands are divided into their subclasses based on the variation in their structure. While both A and B subclass ligands contain the extracellular receptor binding globular domain, the ephrin-A molecules are glycosylphosphatidylinositol (GPI) linked to the exoplasmic leaflet of the plasma membrane (Pasquale, 2005). The ephrin-B molecules however are transmembrane molecules consisting of a transmembrane domain containing conserved tyrosine residues and a C-terminal PDZ domain-binding motif (Liang et al., 2019).

The Eph RTK family does not fall into the conventional receptor ligand signaling mechanism, where the terms "receptor" and "ligand" are somewhat artificial. Both the Eph and ephrin expressing cells are able to signal and thus can function as both receptors and ligands. Conventional signaling through the Eph receptor following ligand binding is referred to as *forward signaling*, while activation of an ephrin ligand upon Eph receptor binding is considered *reverse signaling* (Bruckner et al., 1997; Binns et al., 2000; Murai and Pasquale, 2003). Furthermore, the Eph-ephrin molecules can mediate their response uni-directionally, through either the Eph or ephrin expressing cell, or bi-directionally, through both Eph and ephrin expressing cells simultaneously as reviewed by Kania and Klein (2016). These interactions can be mediated in *trans*, where opposing cells express the receptor or ligand; or in *cis*, where both the receptor and ligand are expressed on the same cell (Dudanova and Klein, 2011; Falivelli et al., 2013; Yoshida et al., 2017). These receptors and ligands interact as dimers and tetramers and larger clusters, where the clustering of Eph and ephrin molecules is essential

to provoke a specific response within a cell (Davis et al., 1994; Himanen et al., 2001; Xu et al., 2013; Liang et al., 2019). The biological outcomes such as adhesion, de-adhesion, migration, proliferation or differentiation are dependent on the quantitative characteristics of Eph activation, where high levels and low levels of expression/activation can induce opposing biological responses (Batlle et al., 2002; Blits-Huizinga et al., 2004; Hansen et al., 2004; Poliakov et al., 2004; Ojosnegros et al., 2017).

The Eph receptors of both subclasses and the ephrin-B ligands can signal through both tyrosine phosphorylation and ensuing protein-protein interactions, as well as protein-protein interactions through the PDZ motif and SAM domain (Binns et al., 2000; Cowan and Henkemeyer, 2001; Lu et al., 2001; Palmer et al., 2002; Leone et al., 2008; Wang et al., 2018; Liang et al., 2019; Baudet et al., 2020). Importantly the ephrin-A molecules localize to lipid rafts/micro-compartments within the plasma membrane and engage transmembrane proteins, such as caveolins, neurotrophin receptor p75 and intracellular Src family kinase dependent signaling (Davy et al., 1999; Davy and Robbins, 2000; Lim et al., 2008). The signaling through Eph-ephrin interactions is essential for a number of developmental and pathophysiological processes (Boyd et al., 2014; Kania and Klein, 2016), including cell attachment, spreading, migration, tissue boundary formation, cellular differentiation, stem cell niche maintenance and proliferation, axon guidance, neural plasticity, somatogenesis, angiogenesis, vasculogenesis, hematopoiesis, immune cell function, cancer tumorigenicity, tissue repair, skeletal development and homeostasis (Kullander and Klein, 2002; Cramer and Miko, 2016; Kania and Klein, 2016; Yang et al., 2018a; Darling and Lamb, 2019; Alfaro et al., 2020; Buckens et al., 2020; Fernandez-Alonso et al., 2020; Giorgio et al., 2020; Vreeken et al., 2020).

THE ROLE OF EPH-EPHRIN SIGNALING WITHIN THE MESENCHYMAL LINEAGE AND CONTRIBUTION TO SKELETAL DEVELOPMENT AND HOMEOSTASIS

The skeleton is formed through two distinct processes termed intramembranous and endochondral ossification. MSC are essential for both processes. Cranial neural crest and the direct differentiation of MSC into osteoblasts contribute to intramembranous ossification. A portion of the clavicle and the cranium are formed by intramembranous ossification. The more complex process of endochondral ossification contributes to the formation of posterior part of the skull, axial and appendicular skeleton. In simplistic terms, during endochondral bone formation MSC condensations contribute to the formation of a cartilaginous scaffold that is systemically replaced to form bone. However, this is a much more involved process which has been elegantly described by Kronenberg and colleagues (Ono et al., 2019). Skeletal integrity is maintained throughout the lifetime of vertebrates through the tightly regulated process of bone homeostasis which takes place within the basic multicellular unit (BMU) or bone remodeling unit (BRU). This process predominantly relies on many cell types maintaining the delicate balance between bone formation and bone resorption (Arthur et al., 2013b; Sims and Martin, 2014) (**Figure 2**).

Identification and Maintenance of MSC Through Eph-Ephrin Signaling

MSC are a desirable source of cells to use in bone tissue engineering applications due to their accessibility, differentiation potential and immune-modulatory effects (Arthur et al., 2009b; Nguyen et al., 2013; Wada et al., 2013). Notably, numerous MSC populations that contribute to the skeletal stem cell niche have been identified using mouse *in vivo* studies (Chan et al., 2015; Crane et al., 2017). A comprehensive review of genetic mouse studies identifying the bone marrow stem cells niche and the translational relevance to human stem cell biology has been described (Chen et al., 2017). Human MSC populations are predominantly referred to as MSC or bone marrow stromal/stem cells (BMSC) and are defined based on three criteria proposed by The Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy. These include: (1) that isolated cells are plastic adherent in culture: (2) that >95% of the cells express the following markers CD73 CD90, and CD105, and >95% of the cells lack the expression of CD14 or CD11b, CD79a or CD19, CD34, CD45, and HLA-DR: and (3) that the cultured MSC have the ability to differentiate into osteoblasts, adipocytes and chondroblasts (Dominici et al., 2006). However, these criteria are an oversimplification of MSC-specific populations and are inadequate indicators of stemness. Other markers have been identified with the capacity to purify clonogenic MSC which exhibit multi-differentiation potential, hematopoietic support and self-renewal capacity *in vitro* and *in vivo*, based on their high cell surface expression of NGF-R, PDGF-R, EGF-R, IGF-R, CD49a/CD29, STRO-1, CD146, and CD106 (Gronthos and Simmons, 1995; Gronthos et al., 2001, 2003; Dennis et al., 2002; Shi and Gronthos, 2003; Sacchetti et al., 2007).

The Eph-ephrin molecules have also been implicated in MSC biology (Alfaro et al., 2020). Certainly comparative analyses studies have identified upregulated levels of EphA2 expression in umbilical cord MSC, compared to MSC derived from other tissue sources and human dermal fibroblastic cells, suggesting that EphA2 may be an unique biomarker characterizing tissue specific MSC (Brinkhof et al., 2020). It is important to note that during cell culture a number of MSC biomarkers are downregulated rapidly coincident with a correlative increase in expression of osteogenic maturation associated genes (Gronthos et al., 2003). While the expression of EphA3 in endometrial MSCs can be dependent on oxygen levels during culture conditions (To et al., 2014). Furthermore, cell passage and cellular aging also contribute to the ability of MSC to adequately differentiate toward the osteogenic lineage (Tanabe et al., 2008). This is an important issue with respect to tissue engineering, which requires clinical grade and scale up of MSC numbers for therapeutic applications. Multiple groups have endeavored to address this issue by investigating the differential gene expression profile of short- and long-term passaged human BMSC (hBMSC), identifying EphA5 among other molecules to be up-regulated during late passages (Tanabe et al., 2008; Yamada et al., 2013). It

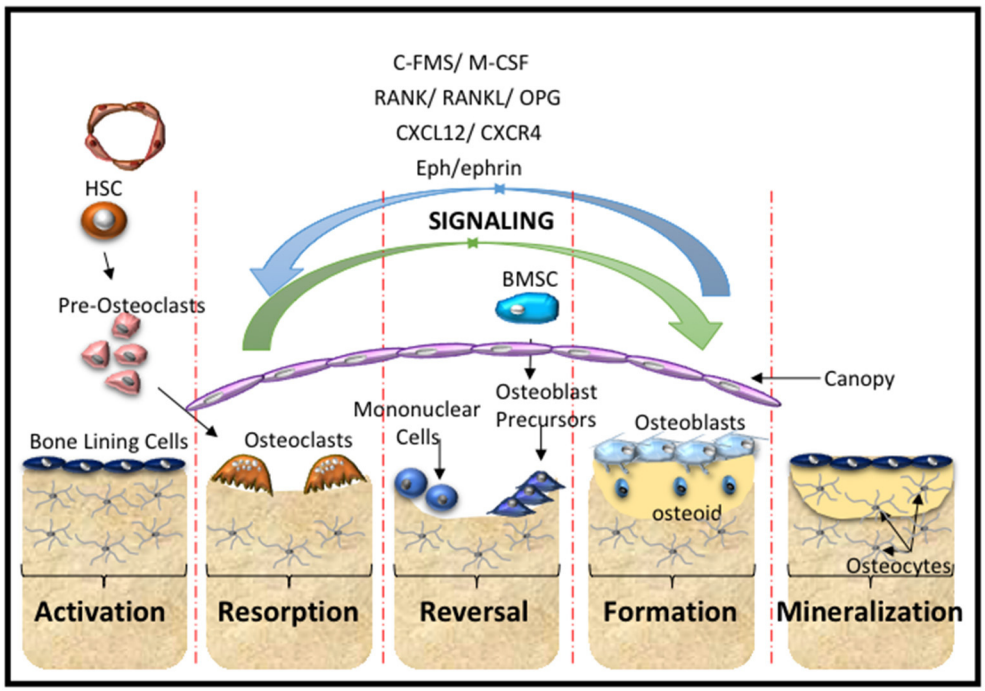

FIGURE 2 | Bone Remodeling. A schematic overview of the cells and main molecular processes involved during the stages of bone remodeling starting with the activation stage, where hematopoietic stem cells (HSC) give rise to pre-osteoclasts of the myeloid lineage that are recruited to the injury site. This is followed by the resorption stage where the pre-osteoclasts undergo maturation and fusion to form mature multinucleated osteoclasts that resorb the bone matrix. The reversal stage sequesters bone marrow stromal stem cells (BMSC) and osteoblast progenitors to the repair site, which is followed by the formation stage, where the bone matrix is synthesized and osteoid is laid down. The mineralization stage involves the mineralization of the osteoid and regeneration of new bone. These cellular responses are mediated by molecular interactions and signaling cascades. The major contributors include the C-FMS, M-CSF, the RANK/RANL/OPG axis, CXCL12/CXCR4 signaling and Eph-ephrin communication.

was proposed that EphA5 mediates inhibitory signals observed in long-term cultures that led to the deterioration of hBMSC differentiation capacity. Therefore, EphA5 may be a negative regulator of hBMSC osteogenic differentiation (Yamada et al., 2013). Subsequent overexpression and siRNA studies support this role for EphA5 and further propose that EphA5 signaling may have a dual role in growth regulation of hBMSC and may also be a potential candidate for replicative senescence (Yamada et al., 2016). Indeed, it was recently proposed that members of both the A and B subclass Eph-ephrin molecules are able to influence MSC survival and adherence *in vitro* (Alfaro and Zapata, 2018).

The B-subclass also contribute to MSC-like populations derived from dental and bone marrow tissues (Stokowski et al., 2007; Arthur et al., 2009a, 2011). Numerous EphB-ephrin-B molecules are expressed by human dental pulp stem cells (hDPSC) within the perivascular niche and the surrounding tissue. Both EphB and ephrin-B molecules play a functional role regulating cell attachment and spreading, and inhibiting cell migration (Stokowski et al., 2007). In the context of an *ex vivo* tooth injury model it was further confirmed that ephrin-B1 activation of EphB molecules expressed by hDPSC was important for MSC niche maintenance under steady-state conditions (Arthur et al., 2009a). Similarly, the B-subclass have also been identified in hBMSC where EphB-ephrin-B communication mediated through *reverse signaling* inhibited hBMSC attachment and spreading, while *forward signaling* promoted migration (Arthur et al., 2011). *Reverse signaling* through ephrin-B molecules is also important for hBMSC chondrogenic and osteogenic differentiation (Arthur et al., 2011). Collectively, these findings demonstrate the importance of Eph-ephrin communication in MSC niche maintenance and differentiation capacity in response to injury of mineralized tissues.

Contribution of Eph-Ephrin Signaling to Chondrogenesis

Pioneering studies have identified the importance of spatial localization of EphA-ephrin-A signaling (EphA4, EphA7, ephrin-A2, ephrin-A3, ephrin-A5) within the earliest stages of skeletal development (Wada et al., 1998, 2003; Stadler et al., 2001; Lorda-Diez et al., 2011) (**Figure 3A**). During the early stages of endochondral ossification, the outer cells of the mesenchymal condensation form the perichondrium, which display overlapping expression of ephrin-A3 and EphA7. EphA7, positively regulated by Hoxa13, subsequently communicates with ephrin-A3 to demarcate the perichondrial boundary (Stadler et al., 2001). Within the developing avian limb bud, ephrin-A2, localized predominantly to the proximal-intermediate regions, regulates the "position-specific" affinity of limb mesenchymal cells, while also contributing to cartilage patterning within the

limb (Wada et al., 2003). Whilst, Eph-ephrin communication is critical for spatial localization, down-stream EphA4 forward signaling has also been shown to contribute to post-natal body growth through the regulation of insulin growth factor (IGF-1). Thus global deletion of EphA4 results in smaller epiphyseal growth plates and short stature and associated low levels of plasma IGF-1 (Jing et al., 2012). The EphA receptors have also been identified within the superficial to middle zone during articular cartilage growth using laser capture microdissection. However, this study did not elaborate further on which receptors were differentially expressed (Lui et al., 2015). Several of these molecules are now being addressed in the context of cartilage related defects, which will be discussed in Section Pathological Conditions Attributed to Alterations Within the Bone Microenvironment in Response to Eph-Ephrin Function of the review.

Recently, the B-subclass of Eph-ephrins has also been implicated in the growth of articular cartilage (Lui et al., 2015). The ephrin-B1 molecule is the only known family member to be associated with a human skeletal phenotype. In humans, loss of function mutations in the *EFNB1* gene result in cranial defects such as frontonasal dysplasia and coronal craniosynostosis (Twigg et al., 2004; van den Elzen et al., 2014). Associated skeletal defects include asymmetrical lower limb shortness and unequal arm span to total height ratio (van den Elzen et al., 2014), which are dependent on correct chondrogenesis and growth plate function. Polydactyly, a cartilage segmentation defect, was also observed in humans (Wieland et al., 2004). The global deletion of ephrin-B1 in mouse causes perinatal lethality and other defects including abnormal cartilage segmentation, ossification pattern (Compagni et al., 2003); and perichondrium maintenance (Davy et al., 2004). Furthermore, abnormalities in cartilage segmentation within the wrist and ribs during embryonic development and in adult ephrin-B1 null mice have also been described (Compagni et al., 2003; Davy et al., 2004). More recently, ephrin-B1 was also identified to be important for growth plate formation (**Figure 3A**). The targeted deletion of ephrin-B1 under the control of the *Osterix* promoter (*Osx:cre-eB1*$^{-/-}$) resulted in developmental growth plate defects in *Osx:cre-eB1*$^{-/-}$ mice when compared to *Osx:cre* controls (Nguyen et al., 2016b). Notably, osterix is expressed by pre-hypertrophic chondrocytes within the growth plate as well as osteogenic progenitors (Oh et al., 2012). In accord with these observations, *in vitro* studies have also noted that ephrin-B1 *reverse signaling* enhances the chondrogenic potential of hBMSC (Arthur et al., 2011), where it contributes to the regulation of the fracture repair process (Arthur et al., 2020).

Interestingly, it appears that the loss of ephrin-B2 also under the control of the *Osterix* promoter, resulted in a strikingly different phenotype. These *Osx1Cre:Efnb2*$^{\Delta/\Delta}$ mice displayed an increase in trabecular bone volume, growth plate remnants and abnormal osteoclasts within the growth plate during skeletal development, which was resolved by 6 weeks of age (Tonna et al., 2016). This observation was attributed to the dependence of ephrin-B2 signaling for the correct production of cartilage degrading enzymes and subsequent endochondral ossification, which then allowed for the correct attachment of osteoclasts and also osteoblasts to the chondro/osseous junction (Tonna et al., 2016) (**Figure 3A**). During postnatal bone development of the secondary ossification center, IGF-1 signaling within the inner layer of perichondral cells promotes proliferation and cartilage matrix degradation (Kozhemyakina et al., 2015). Moreover, IGF-1 increased ephrin-B2 production, which stimulated VEGF expression and subsequent vascularization (Wang et al., 2015). Notably the conditional deletion of ephrin-B2 under the control of the Collagen Type 2 promoter, which has been proposed through lineage tracing studies to be expressed prior to Osterix (Ono et al., 2014), did not result in obvious growth plate defects. Rather these conditional ephrin-B2 knockout mice displayed a defect in the trabecular bone in the metaphysis and epiphysis (Wang et al., 2020). The authors propose that during skeletal development ephrin-B2 expressed by Collagen Type 2 expressing cells contribute to the transdifferentiation of chondrocytes to osteoblasts (Wang et al., 2020). This suggests that in addition to normal endochondral ossification, a proportion of chondrocytes can transdifferentiate into osteoblasts within ossification centers. This is a relatively new area of investigation that is gaining momentum (Yang et al., 2014; Zhou et al., 2014); although the concept still requires further examination. However, it is clear that while ephrin-B1 and ephrin-B2 are structurally similar their function can vary greatly depending on their spatial and temporal expression and interaction with cognate receptors (**Figure 3A**).

Contribution of Eph-Ephrin Signaling to the Osteogenic Lineage

The seminal work conducted by Matsuo et al. in 2006 demonstrated that EphB4, expressed by osteoblasts, and its cognate ligand, ephrin-B2, expressed by osteoclasts signal bi-directionally acting as mediators of bone homeostasis (Zhao et al., 2006). This inspired a body of work examining the importance of Eph-ephrin communication during skeletal development, homeostasis and skeletal repair (Edwards and Mundy, 2008; Martin et al., 2010; Matsuo and Otaki, 2012; Sims and Walsh, 2012; Arthur et al., 2013a; Sims and Martin, 2014; Tonna and Sims, 2014; Rundle et al., 2016) (**Figure 3B**). The research in this field has predominantly focused on the B-subclass Eph-ephrin molecules and the communication between EphB4-ephrin-B2. However, a number of EphA molecules are also expressed within the osteogenic population, including EphA1, A2, A3, A4, and A7 (Zhao et al., 2006; Irie et al., 2009; Matsuo and Otaki, 2012; Stiffel et al., 2014). While EphA4 is important for limb development, chondrogenesis and cranial development, EphA2 has been directly implicated in osteogenic function, inhibiting osteogenesis through RhoA signaling (Zhao et al., 2006; Irie et al., 2009; Ting et al., 2009; Matsuo and Otaki, 2012; Stiffel et al., 2014).

EphB4-Ephrinb2 Communication Within Skeletal Tissue

Our understanding of EphB4-ephrin-B2 communication has expanded over the last 15 years from cell-heterotypic interactions between cells of the osteogenic lineage and osteoclastic cells to cell-homotypic interactions within the osteogenic lineage. The field has utilized the knowledge of Eph-ephrin function

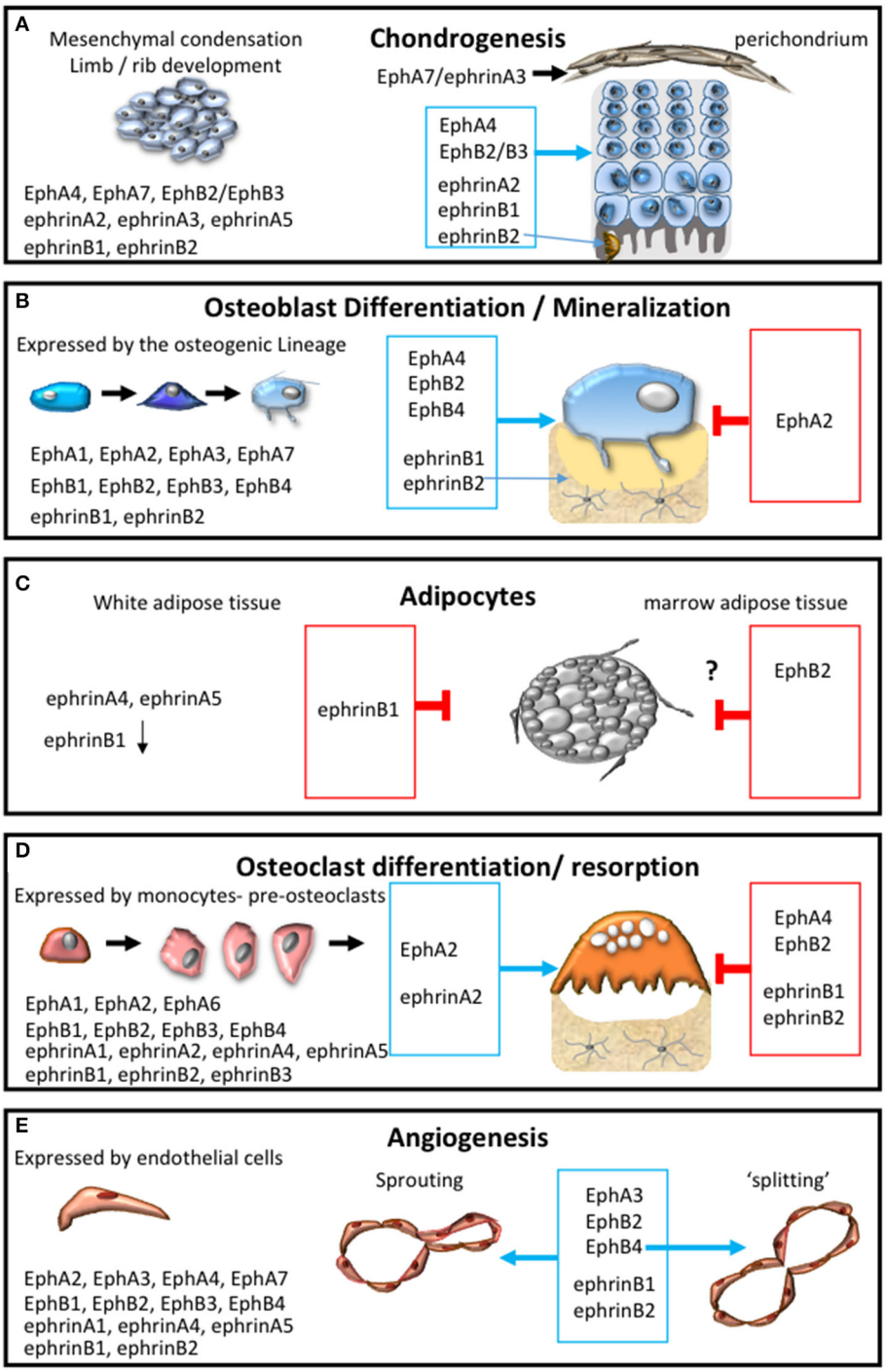

FIGURE 3 | Eph-ephrin communication within the bone microenvironment. The expression profile of A and B subclass Eph and ephrin molecules and their influence on: **(A)** chondrogensis and cartilage formation; **(B)** osteogenic differentiation and mineral formation, **(C)** adipocyte function within white and marrow adipose tissue; **(D)** osteoclast formation, differentiation and resorptive function; **(E)** during the processes of angiogenesis including adhesion, migration, sprouting and intussusceptive "splitting" angiogenesis. The permissive signal is represented in the blue box and the inhibitory response is represented in the red box.

from other biological systems and their association with diverse signaling networks (Arvanitis and Davy, 2008), to understand the molecular interactions of cell surface signaling pathways within skeletal tissue (Lindsey et al., 2018). It is clear that EphB4 *forward signaling* is required for bone formation under steady-state (Zhao et al., 2006) and trauma induced conditions (Arthur et al., 2013a).

Mechanistically, inhibiting EphB4-ephrin-B2 interactions within the osteogenic population reduces the mineralization

potential of mouse stromal cells in a dose dependent manner by parathyroid hormone 1 receptor (PTHR1) (Allan et al., 2008). These observations suggest that ephrin-B2-expressing osteogenic cells are responsive to parathyroid hormone-related protein (PTHrP)/ PTH mediating homotypic interactions presumably with EphB4 and potentially EphB2 to stimulate osteoblast maturation and function (Allan et al., 2008). The N-terminus of PTHrP has been attributed with roles in calcium homeostasis and osteogenic function among other roles. However, it is also evident from mouse knock-in studies that the mid-regional, nuclear localization sequence (NLS) and C-terminus of PTHrP are also essential for osteogenesis (Toribio et al., 2010). These regions influence skeletal mineralization in part through the regulation of ephrin-B2 within the osteogenic lineage (Toribio et al., 2010). Moreover, administration of PTH in the presence of EphB4 blocking peptide to inhibit EphB4-ephrin-B2 interactions, resulted in a multifaceted response in both osteoblasts and osteoclasts *in vitro* and *in vivo* (Takyar et al., 2013). Inhibition of EphB4 mediated signaling reduced the expression of mature osteoblast and osteocyte markers *in vitro*, while osteoblast numbers and activity were increased *in vivo* correlating to a decrease in trabecular number. Collectively the findings suggest that PTH mediated EphB4 *forward signaling* within the osteogenic lineage is important for the later phases of osteoblast differentiation (Takyar et al., 2013).

Furthermore, it is well established that IGF-1 signaling is necessary for PTH stimulation of bone formation (Bikle et al., 2002; Bikle and Wang, 2012). Indeed it has been demonstrated utilizing global IGF-1 knockout mice and complementary *in vitro* co-culture studies using blocking peptides that IGF-1/ IGF-IR signaling mediated through ephrin-B2-EphB4 heterotypic interactions promoted osteoblast and chondrogenic differentiation (Wang et al., 2015). However, while the majority of these studies have focused on EphB4 signaling during osteogenic differentiation, a recent report identified that ephrin-B2 *reverse signaling* is also important for secondary mineralization (Vrahnas et al., 2019). The bone is mineralized through two sequential phases, known as primary mineralization at the calcification front, which is a rapid process (∼60–65% mineralization in ewes). This is followed by secondary mineralization, involving the gradual maturation, accumulation and quality of mineral (Bala et al., 2010). Assessment of an osteocyte specific ephrin-B2 conditional knockout mouse found that the mice developed brittle bones. This was attributed to an acceleration of secondary mineralization resulting in increased mineral and carbonate accrual mediated by enhanced autophagic flux (Vrahnas et al., 2019). This novel finding demonstrates that B-type Eph and ephrin molecules are required for various processes during osteogenesis.

EphB2-EphrinB1 Communication Within Skeletal Tissue

The EphB2 high affinity ligand, ephrin-B1, is expressed by different human MSC-like populations and is a potent mediator of mineralization in both dental (Arthur et al., 2009a) and bone tissues (Arthur et al., 2011), skeletal development (Xing et al., 2010; Nguyen et al., 2016b), homeostasis (Arthur et al., 2018) and trauma (Arthur et al., 2020). Mechanical loading is essential for the maintenance of skeletal integrity, mechanical loading experiments using the tibia identified up-regulation of both EphB2 and ephrin-B1 when compared to the un-loaded control (Xing et al., 2005; Kesavan et al., 2011). EphB2 up-regulation was exacerbated within newly formed bone of transgenic mice overexpressing ephrin-B1 in committed bone cells, suggesting homotypic cellular interactions (Cheng et al., 2013). Since EphB4 expression was unchanged in these studies, mineralization may occur through EphB2-ephrin-B1 interactions independent of EphB4-ephrin-B2 signaling. While EphB2 has been implicated in osteogenesis within the cranial sutures (Benson et al., 2012), it was reported, although not shown, that EphB2 global knockout mice did not develop noticeable differences within the skeleton (Compagni et al., 2003). However, EphB2/EphB3 knockout mice were reported to display patterning abnormalities in the thoracic skeleton (Compagni et al., 2003), indicating some level of functional redundancy within the family. A conditional osteogenic EphB2 knockout study is thus warranted to determine the specific role of EphB2 during axial and appendicular skeletal development and homeostasis.

More is known about the role of ephrin-B1 in osteogenesis, where the global and conditional knockout of ephrin-B1 in osteoblasts results in gross skeletal deformities (Compagni et al., 2003; Xing et al., 2010; Nguyen et al., 2016b). These conditional mice are physically shorter in stature which correlated to reduced bone formation, cortical thickness, and trabecular parameters (Xing et al., 2010; Nguyen et al., 2016b). Conversely, transgenic mice over-expressing ephrinB1 in osteoblast progenitors exhibit enhanced bone formation, within the trabecular and cortical bone, and reduced bone resorption, resulting in an increase in bone mass (Cheng et al., 2013). Importantly, aging (6-month-old) mice lacking ephrin-B1 in the osteogenic population developed an osteoporotic-like phenotype (Arthur et al., 2018). Interestingly, mice with ephrin-B2 knockout using the same promoter reported a significant increase in bone to tissue volume, trabecular number, and thickness at 6 months of age (Tonna et al., 2014). Therefore, it appears that the functions of ephrin-B1 and ephrin-B2 vary considerably during osteogenesis. It appears that the function of these ephrin-B molecules is underpinned by their intercellular interaction with cognate receptors, predominantly facilitated by EphB2 and EphB4, respectively, and subsequent differential intracellular signaling modalities.

Mechanistically, ephrin-B1 intracellular signaling contributes to bone formation in mouse osteogenic cells, mediated through the PDZ domain. The binding of EphB2 with ephrin-B1, results in ephrin-B1 phosphorylation, consequently the ephrin-B1 PDZ domain forms a complex with Protein Tyrosine Phosphatase Non-Receptor Type 13 (PTPN13), Na+/H+ exchanger regulatory factor 1 (NHERF1) and Transcriptional Coactivator With PDZ-Binding Motif (TAZ). TAZ is subsequently de-phosphorylated and released from the ephrin-B1-PDZ complex and translocates to the nucleus inducing the expression of *Osterix* to drive osteoblast maturation (Xing et al., 2010). Recently it was confirmed that hBMSC also utilize the same signaling pathway where EphB2 activation resulted in the de-phosphorylation of TAZ (Arthur et al., 2020).

Involvement of Eph/Ephrin Molecules in Adipogenesis

It is interesting to note that no studies have investigated the contribution of Eph-ephrin signaling within bone marrow adipocytes specifically. However, Zapata et al. recently reported that adipose tissue derived MSC (Ad-MSC) isolated from mice lacking EphB2 increase adipogenesis with minimal influence on osteoblast differentiation. However, Ad-MSC isolated from mice expressing a truncated version of EphB2, which prevents forward Eph signaling while still allowing ephrin reverse signal, resulted in osteoblast differentiation (Alfaro et al., 2020). These observations suggest that perhaps EphB2 forward signaling is important for the inhibition of adipogenesis by MSC. Moreover, Eph-ephrin communication has been reported in white adipose tissue, where ephrin-A4 and ephrin-A5 were found to be a downstream signaling pathway to aldehyde dehydrogenase, which stimulates the development and innervation of white adipose tissue (Shen et al., 2018). Also, ephrin-B1 was identified to be down-regulated in mature adipocytes of obese mice and shown to suppress the adipose inflammatory response (Mori et al., 2013).

Collectively these studies demonstrate that ephrin molecules of both subclasses are implicated in adipocyte biology and therefore investigating the function of Eph-ephrin molecules within bone marrow adipocytes is warranted (**Figure 3C**). Furthermore, it is clear that intercellular Eph-ephrin signaling within the mesenchymal lineage can modulate diverse pathways and biological responses during specific stages of skeletal development and bone homeostasis. However, other resident cells within the bone such as those of the hematopoietic lineage also contribute to skeletal development and homeostasis.

EPH-EPHRIN COMMUNICATION INFLUENCES THE HEMATOPOIETIC SYSTEM WITHIN THE BONE

The HSC niche associates with numerous cell types and location within the bone marrow (Crane et al., 2017). These HSC and their derivatives, the myeloid and lymphoid lineages, are maintained and regulated by the stromal population (Okamoto and Takayanagi, 2019; Tsukasaki and Takayanagi, 2019; Guder et al., 2020). The contribution of Eph-ephrin intercellular signaling between the stromal population and the regulation of these lineages, while important, they are beyond the scope of this review. However, HSC niche maintenance and osteoclast function, both of which are essential for the maintenance of skeletal integrity have been addressed.

Regulation and Maintenance of Hematopoietic Stem/Progenitors by BMSC Through Eph-Ephrin Signaling

We have previously reviewed the stromal–hematopoietic interactions through Eph-ephrin communication, highlighting the role of EphA3-ephrin-A5 and EphB4-ephrin-B2 interactions in BMSC-HSC intercellular signaling (Ting et al., 2010; Nguyen et al., 2015, 2016a). More recently, it has been reported that *EPHA5* and *EPHA7* are expressed by human hematopoietic stem/ progenitor cells. Activation of either EPHA5 or EPHA7 by EPHRIN-A5, expressed by the hBMSC, subsequently stimulates *RAC1* activation and RAC1 target molecule WAVE to enrich the maintenance, migration and adhesion of hematopoietic stem/ progenitor cells (Nguyen et al., 2017). The B-subclass act in a similar manner to the A-subclass in this BMSC-HSC intercellular communication. The conditional loss of ephrin-B1 within the mouse osteogenic population limits the capacity of these osteogenic cells to support the maintenance of mouse hematopoietic stem/progenitor cells (Arthur et al., 2019). Human studies confirmed that EPHB1 or EPHB2 expressing $CD34^{+}$ hematopoietic stem/progenitor cells were responsive to ephrin-B1 stimulation (Arthur et al., 2019). Here it was proposed that the mechanism facilitating this response was mediated in part by *CXCL12* (Arthur et al., 2019), a known critical regulator of hematopoietic stem/progenitor cell function (Greenbaum et al., 2013) (**Figure 4**).

Further investigations into the contribution of EphB4-ephrin-B2 signaling in hematopoietic stem/ progenitor cell mobilization found that EphB4 was expressed by endomucin+ bone marrow sinusoidal endothelium, while ephrin-B2 was expressed by hematopoietic stem/progenitor cells, using a $EfnB2^{H2BGFP}$ reporter mouse (Kwak et al., 2016). Importantly the study reported that the regulation of hematopoietic stem/progenitor cells exiting from the bone marrow was mediated through transendothelial migration, which could be inhibited by using antibodies that blocked EphB4-ephrin-B2 interactions. In the context of cancer therapy, blocking the mobilization of hematopoietic stem/ progenitor cell by inhibiting EphB4-ephrin-B2 communication also resulted in reduced infiltration of the hematopoietic stem/progenitor cells into murine tumor models (Kwak et al., 2016) (**Figure 4**). These observations suggest that manipulation of EphB-ephrin-B signaling has potential therapeutic applications not only in cancer but potentially other diseases and disorders.

The Role of Eph/Ephrin Molecules in Osteoclast Development and Function

Seminal studies identified the expression and importance of a number of A and B subclass Eph receptors and ligands with discrete temporal functions within the osteoclast lineage (Zhao et al., 2006; Irie et al., 2009) (**Figure 3D**).

Contribution of the a Subclass Eph-Ephrin Molecules to Osteoclastogenesis

With regard to the EphA-ephrin-A molecules, osteoclast precursors were found to express EphA2 and ephrin-A2, while EphA4 is specifically expressed by mature osteoclasts. Further investigations revealed that EphA2 and ephrin-A2 are both positive regulators of osteoclast differentiation, where ephrin-A2 mediates down-stream signaling dependent on c-Fos, but not its target molecule NFATc1 (Irie et al., 2009). While it was proposed that ephrin-A2 *reverse signaling* may modulate intracellular calcium signaling through phospholipase Cγ2 (PLCγ2), further investigations are required to confirm these observations. Interestingly, ephrin-A2 was found to be cleaved by

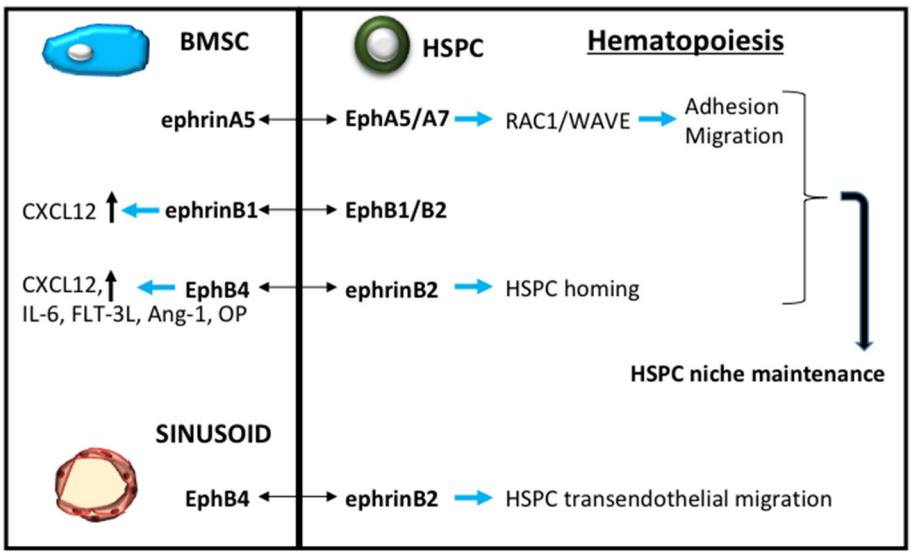

FIGURE 4 | Eph-ephrin contribution to HSC niche maintenance. A schematic demonstrating Eph-ephrin signaling through bone marrow stromal stem cells (BMSC, blue) and sinusoid endothelium to regulate hematopoietic stem/progenitor cell (HPSC, green) maintenance and function.

matrix metalloproteinases (MMPs), where its release enhanced osteoclastogenesis, suggestive of a homotypic interaction between ephrin-A2-EphA2 within the osteoclast lineage. However, as EphA2 was down-regulated when ephrin-A2 was up-regulated, it is plausible that this homotypic interaction takes place between osteoclasts at various developmental states (Irie et al., 2009).

Conversely, EphA4 expression by mature osteoclasts coincides with its function as a negative regulator of osteoclast activity rather than osteoclast formation (Stiffel et al., 2014). Assessment of EphA4-null mice showed reduced trabecular bone volume attributed to osteoclast size and resorption capacity with no change in osteoclast numbers. The molecular mechanisms facilitating this process are thought to be mediated through the activation of the β3-integrin signaling pathway leading to Vav3 activation (Stiffel et al., 2014), where Vav3 is a Rho family GTP exchange factor essential for actin cytoskeletal organization and resorptive activity (Faccio et al., 2005). Like the observations presented for the B-subclass during chondrogenesis and osteogenesis, here we also observe during osteoclastogenesis that EphA receptors, while structurally similar, disseminate diverse functional responses.

Contribution of the B Subclass Eph-Ephrin Molecules to Osteoclastogenesis

Initial studies identifying Eph-ephrin molecules during osteoclast differentiation did not detect the expression of EphB receptors within mouse osteoclast populations (Zhao et al., 2006). However, a recent study identified that *EPHB2* is expressed by human peripheral blood mononuclear cells and during osteoclast differentiation (Arthur et al., 2018). EPHB2 acts as a negative regulator of osteoclast differentiation and function *in vitro*, inhibiting $TRAP^{+}$ osteoclast formation, resorption activity and the expression of *C-FMS*, *CXCR4*, *RANK*, and *CATHEPSIN K* (Arthur et al., 2018).

Conversely, the ephrin-B1 and ephrin-B2 ligands are expressed by mouse osteoclast progenitors and mature osteoclasts (Zhao et al., 2006). Loss-of-function studies determined that ephrin-B1 expressed by the myeloid lineage was a negative regulator of osteoclast differentiation (Cheng et al., 2012). It was proposed that EphB2 activation of ephrin-B1 inhibits NFATc1 expression, while also reducing the phosphorylation of ezrin/ radixin/ moesin (ERM) proteins in mature osteoclasts (Cheng et al., 2012). This protein complex is involved in cytoskeletal rearrangement and cell migration, which are important not only for osteoclast formation but also function. These observations suggest that ephrin-B1 *reverse signaling* plays an essential role for multiple processes in osteoclast biology.

It has also been shown that ephrin-B2 activation in osteoclast progenitors following EphB4 engagement suppresses osteoclast differentiation. This was mediated via the PDZ domain of ephrin-B2, which led to the inhibition of the osteoclastogenic c-Fos-NFATc1 cascade (Zhao et al., 2006; Mao et al., 2011; Wang et al., 2014). Interestingly, *in vitro* mouse osteoclast studies, in which titanium wear particles increased osteoclast formation and function showed that osteoclast activation and the expression of inflammatory markers could be attenuated with the addition of soluble EphB4-Fc, which binds and blocks the receptor binding domain of ephrin-B2 expressed by osteoclasts (Ge et al., 2018). This observation is of particular interest clinically as wear particles can induce inflammation and subsequent periprosthetic osteolysis in response to aseptic loosening following joint replacement surgery.

The communication between osteoblasts and osteoclasts is well established. It was recently documented that during skeletal development and aging (6 months old mice), mice lacking

ephrin-B1 within osteoprogenitors displayed elevated osteoclast numbers within the secondary spongiosa and cortical bone (Nguyen et al., 2016b; Arthur et al., 2018). However, the lack of ephrin-B1 by osteoblasts, did not result in alterations in osteoclast numbers or function (Xing et al., 2010). These studies suggest that osteogenic progenitors also influence the function of the osteoclastic population. This observation is somewhat juxtaposed to current dogma which proposes that osteoblasts and osteocytes, rather than immature osteogenic populations, regulate osteoclast function (Han et al., 2018). However, immature osteogenic regulation of osteoclast function is also supported by the finding that administration of the EphB4 blocking peptide during PTH treatment enhanced osteoclast function *in vivo* (Takyar et al., 2013). Supportive evidence showed that the response was attributed to an indirect function of EphB4 signaling, where blocking of EphB4 in undifferentiated stromal Kusa 4b10 cells resulted in elevated levels of *Rankl, IL-6* and *Osmr*, known promoters of osteoclast formation (Takyar et al., 2013). Collectively, these studies imply that numerous Eph-ephrin interactions contribute to osteoclast function through distinctive spatially and temporally controlled molecular mechanisms. Further investigations are required to determine whether targeting Eph or ephrin molecules is an appropriate therapeutic approach to treat musculoskeletal conditions that are affected by the dysregulation of osteoclasts.

THE ROLE OF EPH-EPHRIN SIGNALING IN VASCULARIZATION AND ANGIOGENESIS WITHIN THE BONE MICROENVIRONMENT

Endothelial cells form blood vessels, supplying the skeletal tissue with nutrients, hormones, oxygen and growth factors, and are critical to skeletal growth, homeostasis and repair (Peng et al., 2020; Zhao and Xie, 2020). A recent review has highlighted the involvement of Eph-ephrin signaling in different endothelial cell populations (Vreeken et al., 2020), with few studies investigating the role of Eph-ephrin homotypic and heterotypic communication between mesenchymal and endothelial cells, during vascularization (formation of the vasculature), angiogenesis (expansion and remodeling of the vasculature) and capillary formation (Adams et al., 1999; Adams and Klein, 2000; Salvucci and Tosato, 2012). The B-subclass Eph-ephrin molecules have predominantly been implicated in these processes, where EphB4 and ephrin-B2 null mice are embryonically lethal (Wang et al., 1998). More specifically EphB3, EphB4 and ephrin-B1 are located on veins, while ephrin-B1 and ephrin-B2 are detected on arteries, where ephrin-B2 has also been implicated in arterial vasodilation (Stein et al., 1998; Adams et al., 1999; Gerety et al., 1999; Adams and Klein, 2000; Lin et al., 2014).

Endothelial cells and the mesenchyme express numerous Eph receptors and ephrin ligands that act through both homotypic and heterotypic interactions (**Figure 3E**). Vascular structures are also supported by pericytes, otherwise known as mural cells. Pericytes, identified by the perivascular marker CD146, share similar properties to MSC (Covas et al., 2008). These pericytes reside within the basement membrane of the vasculature and are key regulators of vascular maintenance and function through the secretion of angiogenic promoting factors. DPSC located within the perivascular niche have been shown to promote angiogenesis via the secretion of VEGF ligands, stimulating VEGFR2-dependent signaling pathways, which included the activation of ephrin-B2 (Janebodin et al., 2013). Activation of ephrin-B2, through its PDZ domain, has also been shown to control VEGFR2 and VEGFR3 endocytosis and subsequent angiogenic sprouting, lymphangiogenic growth and tumor angiogenesis (Sawamiphak et al., 2010; Wang et al., 2010). In the context of Eph-ephrin signaling the assembly of pericyte-endothelial cordlike structures (**Figure 1**), required for vascularization or remodeling, are reliant on Src phosphorylation-dependent down-stream signaling of ephrin-B2 in endothelial cells following activation by either EphB2 or EphB4 (Salvucci et al., 2009).

Similarly, homotypic communication between endothelial cells promotes the formation of cordlike structures, although this was mediated through EphB2 and EphB4 *forward signaling*, and enhanced CXCL12 endothelial chemotaxis (Salvucci et al., 2006). Endothelial cell migration and angiogenesis can also be facilitated by ephrin-B2 stimulation of EphB receptors and more specifically activating the phosphatidylinositol-3 kinase (PI3 kinase) pathway (Maekawa et al., 2003). Conversely, neovascularization can be facilitated by EphB1 stimulation of ephrin-B1 *reverse signaling*, mediated through the C-terminus and most likely the PDZ domain, and required for endothelial attachment and migration facilitated by integrin αvβ3 and α5β1 (Huynh-Do et al., 2002). Taken together, the processes of endothelial migration, angiogenesis and vascularization utilize both Eph forward and ephrin reverse signaling which appears to be dependent on intercellular communication.

Interestingly, EphB4 has also been identified as an important regulator of intussusceptive angiogenesis (splitting of blood vessels), a dynamic process of non-sprouting angiogenesis. Here it was shown that EphB4 can regulate dose-dependent outcomes of VEGF distribution to skeletal muscle that influenced ERK1/2 signaling down-stream of VEGFR2 to "fine tune" endothelial proliferation and circumferential enlargement of vessels without interfering with normal angiogenesis and endothelial migration (Groppa et al., 2018). While EphB4-ephrin-B2 communication is important for pericyte-mediated angiogenesis, this communication did not influence pericyte recruitment (Groppa et al., 2018). Notably, this process of intussusceptive angiogenesis was shown in the muscle, however intussusceptive angiogenesis has been reported in skeletal development and implicated in tumor growth (De Spiegelaere et al., 2012). As developmental processes are often recapitulated during repair, investigating intussusceptive angiogenesis following trauma or musculoskeletal disorders may provide new insight on the endothelial contribution of maintaining skeletal integrity.

In the context of the bone microenvironment, Eph-ephrin signaling of both subclasses has been implicated in tumor progression facilitating several processes including cell proliferation, migration, boundary formation and angiogenesis. More specifically, *EPHA3*, which is elevated in both bone marrow

endothelial cells and plasma cells from Multiple Myeloma patients, promotes their adhesion, migration, angiogenesis and invasion (Caivano et al., 2017; La Rocca et al., 2017). Therefore EphA3 may be an appropriate target for the treatment of Multiple Myeloma (Caivano et al., 2017; La Rocca et al., 2017). Furthermore, endoglin-expressing endothelial cells were recently identified in the bone marrow during fetal development and during regeneration of the adult bone marrow following insult. It was proposed that these endothelial cells may contribute to angiogenesis, osteogenesis and hematopoiesis through the activation of "angiocrine factors." While IL-33 was predominantly investigated in this study, EphA and EphB molecules were enriched in transcriptome studies of the fetal human regenerative endothelial cells (Kenswil et al., 2018).

With respect to tissue regeneration, bone marrow derived endothelial cells are a desirable source of cells that can promote angiogenesis and tissue repair. It was recently demonstrated through a tissue engineering strategy that modulating the stiffness of fabricated substrates regulated arterial-venous differentiation of bone marrow derived endothelial cells, where the EphB4 venous marker and ephrin-B2 arterial marker were differentially expressed based on substrate stiffness (Xue et al., 2017). Collectively, these studies demonstrate the importance of Eph-ephrin signaling in discrete biological processes to facilitate correct angiogenesis and vascularization. However, there is limited knowledge on the contribution of Eph-ephrin interactions within the endothelial population in postnatal skeletal tissues under steady state or pathological conditions or following trauma, warranting further investigation.

PATHOLOGICAL CONDITIONS ATTRIBUTED TO ALTERATIONS WITHIN THE BONE MICROENVIRONMENT IN RESPONSE TO EPH-EPHRIN FUNCTION

Eph-ephrin communication facilitates numerous processes within the bone marrow microenvironment that contribute to maintaining skeletal integrity. Therefore, the loss of any one of these signaling cascades can have detrimental effects to skeletal pathophysiology. While this review does not focus on the pathophysiology of skeletal malignancies, the Eph-ephrin molecules have been identified and contribute to numerous bone related cancers (Buckens et al., 2020).

Osteoporosis

With an increasing aging population comes an increase in the frequency of bone related diseases such as osteoporosis, which is defined as "a systemic skeletal disease characterized by low bone mass and microarchitectural deterioration of bone tissue leading to enhanced bone fragility and a consequent increase in fracture risk," according to The International Osteoporosis Foundation. Certainly, several studies using hormonally regulated osteoporotic ovariectomy (OVX)-induced bone loss models or similar models, have demonstrated the contribution of a number of the Eph-ephrin family members. One proposed treatment target is the communication between EphA2-ephrin-A2, where administration of 17β-estradiol following OVX in rats mitigated the associated bone loss partially through the suppression of EphA2-ephrin-A2 (Liu et al., 2018). Furthermore, an age-related model of osteoporosis in rhesus monkeys identified a gradual increase in bone mass following 12 weeks of treatment with miRNA-based gene therapy (miR-141). miR-141 targeted the osteoclast population, with no differences observed within several organs that were investigated (heart, liver, spleen, kidney) or metabolic processes (blood glucose or cholesterol levels). The study also identified that miR-141 could functionally target EphA2 within the osteoclast population (Yang et al., 2018b).

Prolonged use of glucocorticoids increases the incidence of osteoporotic fractures. The glucocorticoid-induced osteoporosis mouse model causes down-regulation of EphB4 in osteoblasts and up-regulation of ephrin-B2 in osteoclasts. This response was reversed following the administration of icariin, isolated from the Chinese herb Epimedium. Notably there were significant improvements in bone parameters following 4 weeks of icariin treatment (Huang et al., 2020). EphB4-ephrin-B2 expression is also dysregulated in a diabetes-related osteoporosis model (Wu et al., 2016). Together these observations demonstrate the importance of EphB4-ephrin-B2 intercellular communication in maintaining skeletal integrity. However, in the context of lactation-induced maternal bone loss, where prolactin is known to induce the release of osteoclast driver RANKL, up-regulation of the ephrin-B1 gene was detected, with no change in the expression of ephrin-B2 nor EphB4 (Wongdee et al., 2011). Importantly, the loss of ephrin-B1 within the osteogenic population alone results in an osteoporotic phenotype which is comparable to that observed in OVX-induced osteoporosis. Notably, the conditional loss of ephrin-B1 within the osteogenic population diminished skeletal integrity by attenuating bone formation and enhancing osteoclast numbers and function, which was mediated through EphB2 *forward signaling* (Arthur et al., 2018). This observation, in conjunction with its role in inhibiting osteoclast differentiation, suggests that ephrin-B1 may be a key driver in maintaining skeletal integrity. This is supported by the observation that administration of Alendronate (a bisphosphonate) for 8 weeks enhances the expression of ephrin-B1, EphB1 and EphB3 in the mouse femur. Based on *in vitro* cultures ephrin-B1 was also the highest expressed molecule on both osteoblasts and osteoclasts (Shimizu et al., 2012).

Skeletal Repair

The Eph-ephrin molecules have also been investigated in the context of skeletal repair. Tazaki et al. addressed the bone remodeling process using the autologous transplant of goldfish scales, where the scales are formed by intramembranous ossification and mimic the bone remodeling process (outlined in **Figure 2**). While the data showed considerable variability between donors, the authors suggest that ephrin-B2-EphB was required for the activation of osteoprogenitor proliferation during the first absorption phase. During the formation phase ephrin-EphA4 signaling inhibited the communication between osteoclasts, while ephrin-B2 reverse signaling and EphB forward

signaling were involved in osteoblast activation (Tazaki et al., 2018).

Stabilized femoral fracture studies have highlighted the importance and similar function of EphB4 and ephrin-B1 during the callus formation stage of bone modeling. More specifically the transgenic mouse studies demonstrated that EphB4 overexpression in committed bone cells facilitates callus formation *in vivo* following traumatic injury by enhancing endochondral ossification, while inhibiting osteoclast differentiation (Arthur et al., 2013a). Conversely, the loss of ephrin-B1 within the osteogenic lineage resulted in a delay in callus formation and skeletal repair, with an altered distribution of osteoclasts favoring the calcified cartilage (Arthur et al., 2020). This observation was consistent with an independent femoral fracture study that also suggested the importance of ephrin-B1 during the early stages of fracture repair, with the upregulation of ephrin-B1 at 1 and 2 weeks post fracture and localization with mature chondrocyte marker Collagen type 10 (Kaur et al., 2019).

Notably, ephrin-B2 was also upregulated during the first 3 weeks of fracture repair following a stabilized femoral fracture model, with its protein expression localizing to prehypertrophic chondrocytes, osteoblasts and osteocytes (Kaur et al., 2019). This is an interesting observation as it was recently reported that conditional loss of ephrin-B2 in Collagen Type 2 expressing cells also resulted in a significant delay in fracture repair. However, this study used a nonstable tibia fracture model (Wang et al., 2020). Using this model the authors demonstrated that loss of ephrin-B2 within the Collagen Type 2 expressing cells impaired intramembranous bone formation during fracture repair due to the decline in periosteal progenitors. The loss of ephrin-B2 also impaired endochondral ossification during fracture repair due to the reduction in progenitors and VEGF-induced vascular formation within the periosteum and the invasion front of the callus. In addition, there was a reduction in chondrocyte and osteoblast differentiation within the callus which led to impaired bone formation during the later stages of bone repair (Wang et al., 2020). Furthermore, the authors postulate a role for chondrocyte transformation to osteoblasts, although this requires further investigation. Interesting the formation or function of chondroclasts or osteoclasts was not investigated in this study, which is an essential aspect of endochondral bone formation. Collectively these studies demonstrate the relevance and importance of Eph-ephrin function during chondrogenesis and osteogenesis during skeletal repair. As such, targeting these molecules may be a viable therapeutic approach to treating skeletal trauma.

Osteoarthritis

Osteoarthritis (OA) is a degenerative and debilitating disease of the joints and is the most common form of arthritis. While the etiology is not fully known, both systemic (obesity) and non-modifiable (gender, age, genetics) risk factors influence the progression of OA. The predominant feature of OA is the irreversible degradation of the structural proteins (collagens, proteoglycans) within the cartilage matrix of the articular cartilage, as well as cell death (Heinegard and Saxne, 2011). This loss of tissue results in cartilage thinning between adjacent bones, causing bone erosion, and in conjunction, the subchondral bone is remodeled causing sclerosis. Osteophyte formation (bone spurs) ensues, initially as cartilage outgrowths, which then undergo the developmental process of chondrogenesis/endochondral ossification (Hashimoto et al., 2002).

EphB4-ephrin-B2 communication has also been implicated in both chondrogenic and osteogenic metabolism following OA. In OA patients *EPHB4* gene expression is up-regulated in chondrocytes and in osteoblasts of the subchondral bone, where these osteoblasts have pro-resorption properties (Kwan Tat et al., 2008, 2009). Treatment of these chondrocytes or osteoblasts with ephrin-B2 *in vitro* reduced the expression of catabolic collagen degrading molecules in both the chondrocytes and osteoblasts, and inhibited the resorption activity of the osteoblasts (Kwan Tat et al., 2008, 2009). Furthermore, over-expression of EphB4 within osteoblasts was protective against cartilage degradation, sclerosis of the subchondral bone (Valverde-Franco et al., 2012) and the synovial membrane thickness in mice that had undergone the destabilization of the medial meniscus (DMM) OA model (Valverde-Franco et al., 2015). Interestingly, the loss of ephrin-B2 by chondrocytes instigated an osteoarthritic phenotype with aging alone (Valverde-Franco et al., 2016). Of note, ephrin-B1 has been implicated during the inflammatory processes of rheumatoid arthritis (Kitamura et al., 2008; Hu et al., 2015).

Novel approaches to investigate protein-protein interactions and associations between microRNA and genes are also being utilized to identify disease related targets for OA through the analysis of OA meniscal cells rather than the articular cartilage (Wang et al., 2013). The meniscus is composed of a heterogeneous extracellular matrix and fibroblast-like cells, chondrocyte-like cells, and cells with fusiform morphology (Makris et al., 2011). Among other molecules EphA4 was identified and associated with OA (Wang et al., 2013). Recently it was shown that EphA4 was expressed by articular chondrocytes, osteoblasts, osteocytes, meniscal and synovial cells within injured joints of an intraarticular knee injury model (Stiffel et al., 2020). Supportive *in vitro* studies demonstrated that ephrin-A4 stimulation of EphA4 mediated a pro-anabolic response within articular chondrocytes. While, EphA4 activation within synoviocytes facilitated an anti-catabolic response, the authors suggest that targeting EphA4 signaling may be a potential therapeutic approach to treat OA (Stiffel et al., 2020).

THERAPEUTIC TARGETS OF EPH-EPHRIN SIGNALING

The role of Eph-ephrin molecular interactions and specific signaling modalities in numerous tissues and related cancers has led to the development of multiple therapeutic approaches and targets (Barquilla and Pasquale, 2015; Buckens et al., 2020; Giorgio et al., 2020; London and Gallo, 2020) that could be repurposed for the treatment of musculoskeletal diseases/disorders or carcinomas. The drug-based therapeutics include kinase inhibitors, small molecules, monoclonal

antibodies, antibody-drug conjugates, nanobodies and peptides that predominantly target either the kinase domain or the ligand binding domain of the receptor (Barquilla and Pasquale, 2015; Buckens et al., 2020). Depending on the target site, these approaches utilize either selective-agents, as demonstrated with the development of antibodies or less selective-agents such as kinase inhibitors (Giorgio et al., 2020). In the context of currently available drugs a number of pan-kinase inhibitor drug targets, Dasatinib, Sitravinib (MGCD516), JI-101, and XL647, and one selective drug target, an antibody targeting EphA3, Ifabotuzumab (KB004), are currently in clinical trials, predominantly Phase I trials (Buckens et al., 2020). More specific to the musculoskeletal field, a preclinical study treating osteosarcoma utilized drugs that inhibit receptor tyrosine kinase signaling, Pazopanib and Trametinib. The authors identified that this treatment down-regulated EphA2 and IL-7R, and silencing EphA2 resulted in significant reduction of cell proliferation and migration (Chiabotto et al., 2020).

Researchers are also developing novel strategies to identify and generate therapeutic targets with increasing specificity and efficiency. One such approach is the selection of Phage-displayed accessible recombinant targeted antibodies (SPARTA). This process utilizes *in vitro* phage-display screening followed by multiple rounds of sorting with yeast-display screening and the intravenous injection of the selected phage particles into tumor-bearing mice, where they undergo further selection, recovery and amplification (D'Angelo et al., 2018; Tang et al., 2020). This technique was used to generate anti-EphA5 antibodies that have shown specific targeting of EphA5 expressing lung cancer cells (D'Angelo et al., 2018; Tang et al., 2020). Another approach is the generation of peptide antagonists. The Eph-ephrin specific blocking peptides predominantly target the Eph receptor (Koolpe et al., 2002, 2005; Murai et al., 2003), with limited peptides targeting the ephrin ligands (Tanaka et al., 2010). The majority of these peptides bind to the ligand-binding domain of the Eph receptor limiting ephrin ligand binding and thus inhibiting Eph activation. Researchers have subsequently extended the half-life of existing peptides through the addition of polyethylene glycol polymer. One such example is TNYL-RAW, which blocks the binding of the ephrin ligand to the ligand binding domain of EphB4 (Noberini et al., 2011). Recently a peptide with dual function, specifically targeting EphB4-ephrin-B2 interactions was developed. This molecule, termed bi-directional ephrin agonist peptide (BIDEN-AP), can inhibit ephrin-B2 endothelial cell angiogenic signaling, while also activating EphB4 dependent tumor-suppressive signaling in tumor cells (Xiong et al., 2020). *In vivo* mouse studies confirm a significant reduction in ovarian tumor growth following the administration of BIDEN-AP (Xiong et al., 2020). This approach could be beneficial when targeting a known receptor-ligand pairing responsible for a specific biological function. Based on the current set of tools available to manipulate Eph-ephrin interactions, there is scope and potential for these therapeutic targets to be exploited and repurposed for the treatment of other diseases and disorders including those related to musculoskeletal pathophysiology.

CONCLUDING REMARKS

There is considerable complexity in the intercellular interactions between numerous cell types within the bone microenvironment. Throughout this review we have highlighted the multifaceted Eph-ephrin interactions within and between stromal, hematopoietic and endothelial cell types and with the surrounding extracellular matrix. With the development of appropriate research tools including conditional knockout and transgenic mice, specialized *in vitro* culture systems, unique engineered substrates, soluble Eph and ephrin-Fc fusion proteins, Eph-ephrin inhibitory peptides and functional blocking antibodies, we have a greater understanding of how these cells interact within the bone through Eph-ephrin communication to maintain skeletal integrity. It is clear that the Eph-ephrin family members play a role in many vital biological processes during skeletal development and in maintaining skeletal physiology, where dysregulation can lead to a number of pathophysiological conditions within the musculoskeletal system. Eph-ephrin signaling research has identified potential new drug targets that could be exploited for the treatment of musculoskeletal conditions such as fracture repair, periprosthetic osteolysis or disease states such as osteoarthritis or osteoporosis.

Despite our extensive knowledge in this field, there is still a considerable amount of research required to fully understand the role of Eph-ephrin communication within the bone microenvironment. For example it is clear that the MSC population is highly heterogeneous. Could Ephs and ephrins be used as markers to identify MSC subsets? Certainly, this is already under investigation with the identification of EphA2 within different human MSC populations, and the proposed contribution of EphA5 regulating MSC growth, while numerous B-subclass members have been implicated in MSC niche maintenance. There is limited knowledge on the function of Eph-ephrin signaling within the marrow adipose tissue.

The involvement of numerous Eph-ephrin signaling partners contributing to a particular cellular process in a spatial and temporal manner is a reoccurring theme evident throughout this review. It is yet to be determined how multiple Eph receptors or ephrin ligands are expressed simultaneously over a range of developmental stages to differentially influence biological processes. Overall, Eph-ephrin interactions appear to be required as a mechanism to "fine tune" a myriad of processes required for skeletal development, maintenance and repair.

We are also just starting to appreciate the interaction of Eph-ephrin molecules with up-stream and down-stream signaling targets within resident cells of the bone microenvironment. Currently known targets include IGF-1 and PTH, which interact with Eph-ephrin signaling during osteoblast and osteoclast formation and function; the communication with CXCL12 signaling during hematopoietic niche maintenance; or the interaction with integrin molecules during cell adhesion of endothelial cells or osteoclasts. Knowing these molecular interactions and the associated up- or down-stream signaling pathways provides us with a better understanding not only of

the biology but also the dynamics and fluidity that is required to develop potential therapeutic targets to treat musculoskeletal diseases and disorders. This is already evident with current therapeutic approaches targeting different domains of the Eph receptors and ephrin ligands to treat a range of cancers. Based on current knowledge there is an opportunity to combine and utilize multidisciplinary approaches to repurpose tools and drug targets to influence Eph-ephrin communication as a therapeutic strategy to treat diseases and disorders relating to musculoskeletal tissue.

AUTHOR CONTRIBUTIONS

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