IDO ASSAY TO ASSESS MESENCHYMAL STEM CELL POTENCY OVER MULTIPLE PASSAGES

by

Kejie Rui

(Under the Direction of Luke J Mortensen)

ABSTRACT

FDA-approved mesenchymal stem cell (MSC) clinical therapies have been elusive despite strong evidence of their potential to modulate immune response in preclinical models. This failure stems in part from the loss of immune modulatory activity after the high degree of expansion required to make an adequate dose of MSCs for clinical use, and an inability to monitor MSC immune modulatory potential during the expansion process.

Studies show that indoleamine-2,3-dioxygenase (IDO), which is the first and rate-limiting enzyme that catabolizes tryptophan (a promoter of T-cell activity) into L-kynurenine, can be considered as a critical factor during MSCs suppression of T-cell proliferation.

Therefore, here we investigate MSC immune potency through IDO activity and expression as well as the relationship to MSC morphology, proliferation, and senescence. Our work focuses on the following: 1) the change in IDO activity over passages with a

concurrent change in cell proliferation rate; 2) the potential of a correlation between IDO expression and cell morphology; 3) using a morphological imaging approach to separate high IDO potency and high proliferation MSCs during culture expansion.

The success of our future work could provide key insights into potency metrics that would allow for monitoring and selection of high immune potency MSCs. This would significantly increase the efficacy of MSC therapies and provide much-needed relief to patients suffering with immune diseases.

Keywords: Mesenchymal Stem Cells (MSCs), Indoleamine-2,3-dioxygenase (IDO), Morphology, Proliferation, Immunomodulatory Potency

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Kejie Rui

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by

Kejie Rui

Major Professor: Luke J Mortensen
Comittee : James N Warnock
Ross Marklein
Cheryl Gomillion

Electronic Version Approved:

Ron Walcott
Interim Dean of the Graduate School
The University of Georgia
May 2020

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CHAPTER 1
LITERATURE REVIEW

1.1 Brief History of MSCs

The existence of a stromal precursor giving rise to mesodermal cells in the bone marrow was originally theorized by Cohnheim [1] and Maximow [2] in the nineteenth century where they mentioned a reservoir of stromal cells in the bone marrow that were involved in the natural healing response and hematopoiesis. Later, Friedenstein [3] and colleagues first discovered and isolated a type of adult stem cells with an adherent fibroblast-like population in the bone marrow. They also observed that MSCs can differentiate into adipocytes, chondrocytes, and osteocytes which was later confirmed [4]. In the late 1980s, Maureen Owen[5] and Arnold Caplan [6] elaborated on Friedenstein's initial work and proposed the existence of an adult stem cell that was responsible for mesengenesis. Owen et al. further characterized the marrow stroma and illustrated the heterogeneity of the population [5, 7, 8, 9] while Caplan and colleagues hypothesized that a subpopulation of the marrow stroma was developmentally linked to the mesenchymal tissues he had been studying during chick embryogenesis [6, 10]. Later, the subtype of marrow stromal cells involved in the process of mesengenesis was named as mesenchymal stem cells [6].

As methods for isolating and culturing MSCs improved, there was an explosion in the investigation of MSC differentiation pathways and potential immune modulation uses. MSCs were first considered to be stromal progenitor cells in the bone marrow and were originally hypothesized to serve one primary role in their undifferentiated state: replenishment of stromal tissue in the bone marrow. In addition, MSCs and their stromal progeny

perform a number of other functions in the bone marrow especially in the support of hematopoiesis and hematopoietic stem cells through cell-cell mediated interactions and the secretion of soluble factors [11]. MSCs have since been shown to directly influence the innate and adaptive arms of the immune system [12], enhance proliferation of epithelial cells [13], and promote neovascularization of ischemic tissues [14].

In summary, MSCs play important roles in cell life not only by differentiating into various cell types used in tissue regeneration but also for disease treatment. Further investigation of MSCs is needed for the development of the safe and effective MSC applications.

1.2 MSC Definition

With the increase in studies on MSCs, in order to compare the experimental results among different laboratories, in 2006, the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy proposed a set of standards to define human MSCs for laboratory-based scientific investigations and pre-clinical studies [15]: (1) MSCs must be plastic-adherent when maintained in standard culture conditions using tissue culture flasks; (2) Of the MSC population, $\geq 95\%$ must express CD105, CD73, and CD90, as measured by flow cytometry. In addition, these cells must lack the expression ($\leq 2\%$ population) of CD45, CD34, CD14 or CD11b, CD78a or CD19, and HLA class II; (3) The cells must be able to differentiate into osteoblasts, adipocytes, and chondroblasts under standard in vitro differentiation conditions.

1.3 Biological Function of MSCs

In order to better classify and summarize the biofunction of MSCs, we break down the functional properties of in vitro MSCs needed for the creation of a cell therapeutic into their proliferative capacity, their multipotency, their homing and migration properties, their secretory or trophic functions, and their immunosuppressive functions [16].

1.3.1 MSC Self-renewal and Expansion

In order to generate enough cells to create a therapeutic, MSCs must be able to proliferate in vitro (i.e. self-renew) while retaining a fibroblast-like morphology, multipotency, and secretory profile [17, 18]. However, MSCs have a limited expansion capability, and as they become exhausted, the cells no longer proliferate. MSCs' decreased cell proliferation appears as archetypical cell senescence with progressive loss of proliferation and cell cycle arrest [19], and senescence varies among the MCSs derived from different tissues or isolated from different donors [20, 21]. Notably, even with senescence, beneficial phenotypes are retained in many cases. However, the significance of senescence in terms of other MSC functions and therapeutic potency is unclear [22]. Thus, concerns should not only focus on MSCs expansion but also on senescence significance.

1.3.2 Multipotency Function

A key feature traditionally defining MSCs is their differentiation into adipocytes, chondroblasts, and osteoblasts under a definite stimulation in vitro. Their multipotency function was long thought to be therapeutically practical for tissue regeneration [23, 24]. Significant progress has been made in the use of MSCs to treat critically sized bone defects (injuries that are too large to heal on their own) [25, 26], which could find broad clinical use. For cartilage, many studies have investigated the creation of differentiated chondrogenic cells and scaffolds for repair of cartilage [27, 28, 29]. For both of these uses, Seeding MSCs on 3-D bio-material scaffolds could improve cell-cell interactions and facilitate cell differentiation into skeletal-related tissues [30, 31]. When intravenously injected, MSCs have also shown the potential to improve bone disorders such as osteoporosis [32], osteogenesis imperfecta [33], and hypophosphatasia [34]. However, little engraftment has been detected, suggesting that other mechanisms are contributing to the observed therapeutic effects. Since MSC therapies have seen promise in other degenerative diseases where engraftment would not be beneficial [23, 24], this suggests the possibility of a common MSC therapeutic mechanism after systemic administration. Although some clinical studies have shown therapeutic benefits with tissue-engineering use based on MSCs [35], the development of using MSCs

for tissue engineering is still in its infancy, and more research needs to be conducted on their multipotency function.

1.3.3 Trophic Supporting Function

Because MSCs produce growth factors, chemokines, interleukins (ILs), and extracellular matrix molecules, one in vivo role and potential therapeutic use of MSCs has been suggested to be as supporting cells for hematopoiesis. Hematopoietic stem cells (HSCs) co-transplanted with MSCs enable the success of HSC transplants in vivo [36]. This likely occurs through the production of pro-hematopoietic cytokines like IL-6 and stromal-cell-derived factor-1(SDF-1) [37, 38], as well as cell-cell engagement through the Notch signaling pathway [39]. This is why MSCs are considered to have the trophic function to regulate homeostasis in vivo by supporting the maintenance, expansion, and differentiation of HSCs. Beyond the ability to sustain HSC homeostasis and bone tissue, the MSC trophic function might be beneficial in favoring tissue healing and regeneration in other organs [38, 40].

1.3.4 Homing/Migration Functions

The MSC fate resulting from systemic adoptive transfer could occur with (1) passage/location in non-specific tissues, (2) homing into native niches, or (3) migration into damaged and/or diseased tissues [41]. However, it is not thus far well-established how and under what kind of conditions MSCs might survive or be eliminated from the host [42, 43]. Studies on MSC homing/migration function become meaningful when they involve preclinical models because some results agree with clinical findings regarding the biodistribution of MSC post-adoptive transfer [44, 45]. However, MSC homing/migration is required for MSCs to reach their target sites when delivered systemically [46, 47]. Recent advances in molecular biology have augmented the opportunities for adjustment and manipulation of stem cell functionally and are mainly exerted by means of genetic engineering [48]. In addition, improving endothelial adhesion properties is another valid strategy to improve MSC homing [47]. While MSC homing/migration is rather less important for local adop-

tive transfer of MSCs [46, 43, 47] or in cases where the cells do not seem to require homing to target tissues [49], many clinical trials using MSCs would benefit from increased delivery of the cells to a site of disease. Thus, further investigations are needed to properly define MSC homing/migration in vivo and to enhance the efficacy of MSC therapy.

1.3.5 Immunosuppression Function

Lastly, the main point of this project is the validated therapeutic value of MSCs in various immune disorders. According to reported data from the National Institutes of Health ClinicalTrials.gov website (https://www.clinicaltrials.gov/), MSC therapy is used for immunomodulation mostly in immune rejection and autoimmunity, including conditions such as in HSC transplantation, solid organ transplantation, Crohn's disease (CD), rheumatoid arthritis (RA), and systemic lupus erythematosus (SLE) [50, 51]. An early MSC clinical trial study in 2004 on a 9-year old patient with severe graft vs host disease (GvHD) of the gut and liver confirmed the incredible potential of MSCs as a therapy for immune disease in humans. As shown in Figure1.1, an intravenous administration of MSCs significantly lowered the bilirubin and stool samples of the patient over the course of 10 months, which are indicators of liver and gut function, respectively [52]. The outcomes observed within a few days and lasting for at least up to 1-year post-adoptive transfer of MSCs from his mother in this patient showed overall improvement and stability of his condition, which proved the practicability of MSC adoptive transfer. Disease amelioration after MSC adoptive transfer has been substantiated in experimental models of acute kidney injury [53], cardiomyopathy [54], diabetes complications [55], liver cirrhosis [56], and eye diseases such as glaucoma, macular degeneration and retinitis pigmentosa [57], where the primary mechanism of action is likely through reduction of immune response and inflammation. Although increasing evidence seems to indicate that MSCs could revolutionize medicine in patients suffering from immune diseases, the action of the MSC immunosuppression function in vivo still needs extensive research [50, 58].

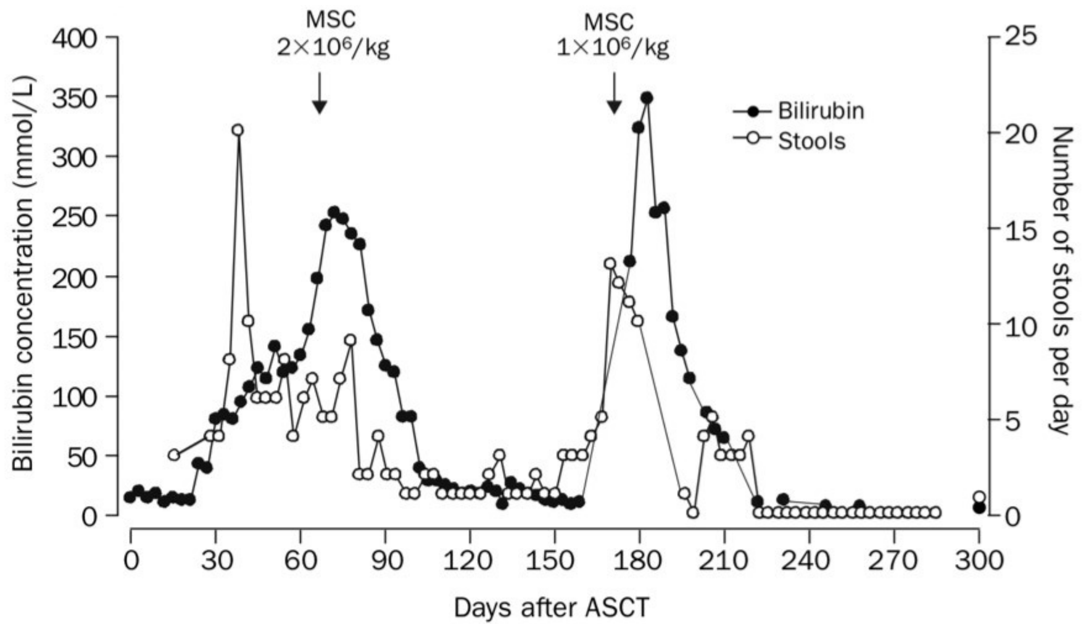


Figure 1.1: Early MSC clinical trial on a patient with severe gut and liver GvHD shows substantial reduction in GvHD symptoms after intravenous MSC administration [52]

1.4 IDO as an MSC Potency Predictor

In 1936, Kotake and Masayama described an enzyme called tryptophan oxygenase constitutively expressed in mammal livers, which was later renamed as tryptophan dioxygenase (TDO). They believed that this enzyme is the solely responsible for the breakdown of L-tryptophan to the catabolite L-kynurenine [59]. However, later studies where mice fed with D-tryptophan could also break it down even if TDO is specific for the L-tryptophan stereoisomer [60]. This confusion went unexplained until 1967 when Yamamoto and Hayaishi [60] discovered another enzyme in rabbit intestine homogenates, indoleamine 2,3-dioxygenase (IDO), that could break down both D- and L-tryptophan. Further investigation on IDO not only demonstrated that this was the enzyme present in various tissues but also found that its expression could be induced by certain infectious agents, lipopolysaccharides, and cytokines such as interferon-gamma(IFN-γ) [61, 62, 63]. Later, studies on IDO's role and function in various autoimmune diseases, transplantation medicine, and cancer-related immunosuppression were boosted [64, 65, 66, 67]. IDO is known to be

more directly involved in the immunosuppressive properties of MSCs and in the suppression of antigen-driven proliferation of T-cells [68, 69, 70].

Tryptophan shortage inhibits T-lymphocytes division, and accumulation of tryptophan catabolites induces T-cell apoptosis and differentiation of regulatory T-cells [71]. IDO catalyzed the first and rate-limiting step of tryptophan catabolism along the kynurenine pathway, and so it acts as a regulator the metabolism of the essential amino acid tryptophan [72]. As two distinct enzymes that catalyze the same reaction, IDO2's affinity for tryptophan is much lower than that of IDO1's. As a result, IDO1 is the target for therapy in a range of clinical settings, including cancer, chronic infections, autoimmune and allergic syndromes, and transplantation [73].

Currently, MSCs have been thought to suppress the immune response through contact inhibition and multiple signaling factors. In addition, IDO expression, which is induced by IFN-γ and catalyzes the conversion from tryptophan to kynurenine, has been identified as a T-cell inhibitory effector pathway in professional antigen-presenting cells [74, 75]. IFN-γ is a potent pro-inflammatory cytokine that is produced by multiple cell types; it plays an important role in both innate and adaptive immune responses and can be considered to be a pathogenic factor related to acute GvHD [76]. It is widely believed that MSCs need a "license" to exert their immunosuppressive function, and IFN-γ is known as a key cytokine capable of providing MSCs with that stimulus [52, 77]. Also, IFN-γ could be produced by a subclass of T cells and then activate macrophages to produce IDO which suggested a relationship between the antiproliferative effect of IFN-γ and IDO induction [78]. With the previous reports about the role of IFN-γ in activating MSCs in vitro [52, 77, 79] and IDO as one of the soluble factors which related to MSC immunosuppression function, the activity, and expression of IDO can be considered as one of the MSC potency predictors. Therefore, understanding and tracking IDO activity and expression according to various conditions will be the main part of this project.

1.5 Proliferation and Senescence

As is well known, prolonged culture expansion leads to the replicative exhaustion/ senescence of MSCs [80]. Cellular senescence is an antitumor genesis process used by replicating cells in conjunction with cell death programs to prevent malignant transformation. Previous studies have demonstrated that cellular senescence increases the heterogeneity of cell populations and leads to uncertainty in therapies' outcomes [81]. Also, long-term in vitro culture expansion may also cause lower proliferation rate, which affects the large-scale cell manufacturing. Replicative senescence on MSCs has shown induction of alterations in MSC functionality [19, 21, 82, 83, 84, 85, 86], but there is still a huge gap to understand the effect of these alterations on MSCs' interaction with immune responders and responsiveness to inflammation.

Currently, since early-phase clinical trials have demonstrated that MSC infusion is safe, marrow-derived MSCs (BM-MSCs) have been widely used under clinical investigation to test their use in adoptive immunosuppressive cell therapy for auto- and allo-immune disorders [87]. Thus, variations in efficacy attracted more attention because higher efficacy is desired in clinical trials [88, 89].

Besides interindividual variability, the large scale of culture expansion will also cause replicative senescence, which can be considered as a critical culture-related parameter and could influence clinical-grade MSC safety and clinical efficacy [89, 90]. Because senescence is observed in expanding human MSCs [90], it was hypothesized that a long-term culture affects MSC clinical efficacy due to replicative senescence of MSCs. This hypothesis has been supported by clinical findings that late passage MSCs appear to be less effective in ameliorating GvHD than early passage cells [89, 91]. Considering that an average cell dose for most clinical studies is around 1 to 2 million MSCs per kilogram of body weight, most academic cell-processing centers will typically manufacture 5 to 10 doses from an allogeneic donor. However, the amount of industrial-scale expansion of MSC-like

cells can be as high as 10,000 to 1,000,000 doses per donor [92]. Therefore, MSCs with a high number of cumulative population doubling levels(PDL) are involved in the large-scale expansion.

Considering the senescent and proliferative properties of MSCs during long-term culture expansion, MSCs potency is possible to be affected when made a comparison between higher and lower passage products. This possibility has thus been the main focus of attention in MSC-potency investigation. Further studies associated with proliferation and senescence will provide deeper knowledge on the change in MSC potency over passages.

1.6 Clinical Perspectives

MSCs seemingly broad immune suppressive ability has led to numerous studies on their efficacy in treating devastating animal diseases in animal models as well as rapid translation to clinical trials [93]. In addition to the bone marrow (BM), adherent cells fitting common definitions of the ”MSC” were isolated from adipose tissue, amniotic fluid, peripheral blood, the placenta, and Wharton's jelly [94, 95]. Moreover, MSCs became considered as an excellent candidate for therapy because (1) human MSCs are easily accessible; (2) the isolation of MSCs is straightforward and the cells can expand to clinical scales in a relatively short period of time [96, 97]; (3) MSCs can be bio-preserved with minimal loss of potency and stored for point-of-care delivery [98, 99]; and (4) human trials of MSCs thus far have shown no adverse reactions to allogeneic versus autologous MSC transplants, enabling creation of an inventory of third-party donor MSCs to widen the number of patients treated by a single isolation [100, 101, 102]. Due to these advantages, only a few years after MSCs were identified, human trials were commenced to evaluate the safety and efficacy of MSC therapy.

Although the primary mechanisms of action have not been fully understood, studies have shown that MSCs could protect cells from injury and directly promote tissue regeneration [103, 104]. When administered to treat animals undergoing acute renal failure,

MSCs prevent apoptosis and promote the proliferation of renal-tubule epithelial cells in a differentiation-independent manner [105, 106]. When administered to prevent the onset of type I diabetes mellitus (IDDM), MSCs protect beta-islets from autoimmune attack. Administration after onset showed that MSCs promote temporary restoration of glucose regulation, suggesting protection and repair of damaged islet tissues [107]. In addition to promoting tissue repair directly, MSCs have also been shown to modulate the immune system and attenuate tissue damage caused by excessive inflammation.

MSC regulation of immune responses is especially attractive for autoimmune diseases and other diseases with immune dysfunction and imbalanced immune regulation that otherwise lack therapies. Based on their capability, clinical applications of MSCs indicate a new prospective approach for immune-related diseases treatment. The Clinical Trials database indicated that the trials using MSCs for immune-mediated diseases have increased annually since 2004, especially in 2011(http://clinicaltrials.gov). As of October 18th, 2016, 127 clinical trials have focused on immune-mediated diseases related to MSCs treatment (Figure 1.2), making up approximately one-fifth of the entire number of MSC clinical trials. These trials overlay immune-mediated diseases targeting different organs and translate rejection, as well as some typical autoimmune diseases and HIV.

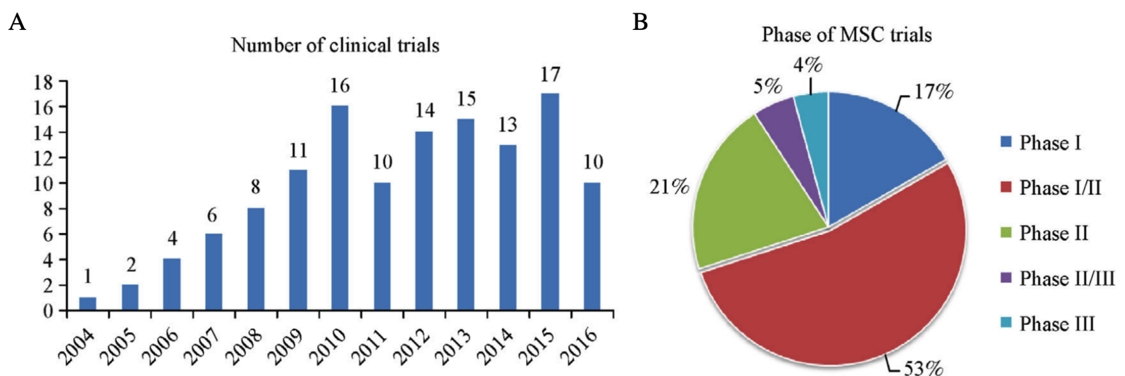


Figure 1.2: Analysis of MSC clinical trials on immune-related diseases
(A) numbers of newly created clinical trials of MSC on immune-related disorders. (B) clinical trials of MSCs classified by phases. [108]

Results from clinical trials showed that MSCs from different sources exhibited parallel therapeutic effects on immune-mediated diseases [109]. The majority of these trials are in Phase I/II (52%); 16% in Phase I for safety studies and 21% in Phase II to investigate efficacy. Only 4% and 5% are in Phase III and Phase II/III respectively (Figure 1.2). Notably, among all these trials only two trials for GvHD are in Phase III which indicates that more studies and significant progress is needed.

1.7 Current Challenges of MSC Therapy

Although numerous studies have been performed to improve MSC clinical trials for almost two decades, little progress has been made on potency metrics to reliably assess and predict the efficacy as part of the clinical trial process [58]. However, this challenge has been acknowledged for many years. For example, a notice published by the FDA in 1993 outlined that cell therapies must have quality control, safety, efficacy, and potency thoroughly addressed before a cell therapy product can be approved for marketing to the public [110, 111]. For general pharmaceutical drugs, it is the standard pharmaceutical practice to assess the potency of a batch of medication that indicates how effectively it will perform. This allows for consistent production of high-quality medications that can be turned for dosing depending on the ailment severity and response rate of the patient. However, unlike standard pharmaceutical drugs, MSCs are served as live therapy which presents the unique and challenging requirements for developing a standard immune potency metric.

In nature, MSCs are heterogeneous, but they can be expanded as adherent cultures. At this point, MSCs are phenotypically well described based on the identification of surface markers proposed by the International Society for Cellular Therapies in 2006 [15]. Although there are common properties of MSCs and those can be considered as the only standards for MSC immune potency assessment in clinical trials, it is also possible that the phenotype and function could be influenced by the age, donor, and culture method. In addition, although this standard method can be considered as an accepted way to identify

MSCs, there is still no way to reliably test or even modify their efficacy. One of the important reasons for a lack of potency metrics is a poor understanding of the main mechanism of action for MSC immunomodulation.

Despite these challenges, MSCs are moving closer to FDA approval for some indications. An Australian company, Mesoblast Ltd, announced in 2018 [112], nearly 70 percent of children with potentially fatal acute GVHD responded to treatment with an experimental stem cell therapy. They believed that 6-month safety and efficacy data from this 55-patient Phase 3 trial may be sufficient for filing for accelerated U.S. approval. Although strong evidence showed the potential of MSCs to modulate immune response in preclinical models currently, no MSC therapy has been approved by the FDA in the United States. This failure may due in part to the loss of immune modulatory activity after the high degree of expansion required to make an adequate dose of MSCs for clinical use and an inability to monitor MSC immune modulatory potential during the expansion process.

Therefore, it is critical for us to understand how MSCs suppress the immune response, and we need to dig deeper into the underlying mechanisms that contribute to these actions.

1.8 MSC Manufacturing and Future Work

Although significant progress has been made in preclinical and clinical studies utilizing MSCs, considerable challenges still remain before MSC therapies can finally move to clinical practice.

First and most important should be the optimization and generalization of parameters related to MSCs before the clinical application to guarantee MSC quality:

(1) *Optimal passage of MSCs to maximize potency* – Many studies have demonstrated that transformation and senescence occur in later passage of MSCs. In particular, Liu's work suggested that cells around passage 3 to 4 showed better features instead of cells just after thawing [113]. Also, freezing steps and cryopreservation will potentially affect the quality and cell functionality during cell manufacturing [114]. According to all these

potential impacts on MSC quality, studies on finding the MSC with higher potency are indispensable by tracking with the in vitro passage as well as the PDL number.

(2) *Optimal route of administration and cell dose* – When it comes to the clinical practice, the route of administration and reasonable dose of MSCs administered should be optimized according to disease type and severity. To date, systemic administration is the main route used for MSC delivery in animal disease models and clinical studies. Due to MSCs' homing and migration function, the number of MSCs homing to target sites for repair might be reduced. This has been proven by a previous study that demonstrated that, during systemic administration, most MSCs become trapped in the liver and lungs instead of the target sites [41]. Therefore, compared with systemic infusion, administration of MSCs to specific target site may bring out better efficacy. Generally, the widely used MSCs dosage is approximately $1 * 10^6$ cells/kg of body weight. However, Kim's work showed the treatment effect may vary with the administration times to GvHD mice [76]. Thus, dose-escalating MSCs to define the optimal cell dose can be considered to figure out the optimal dose of MSCs administered and to consider the types and severity of disease needed to be treated.

With advantages and attractive perspectives of MSCs therapy, large-scale cell manufacturing and MSC production are worthy of consideration based on these parameter optimized MSCs. However, considering the enhanced heterogeneity of MSCs during culturing expansion which likely brings out the inconsistent clinical outcomes [81], the need for identification of high potency populations is also desired before large-scale expansion. Inspired by previous work where morphological heterogeneity following IFN-γ stimulation involved to identify functional subpopulations [115], identification, and separation according to both nucleus and morphological features are considered as a potential strategy for future single-cell characterization.

It is important to have a consensus standard for MSC production during cell manufacturing, so that the results obtained from different clinical trials may be easier to compare. As the better way to manufacture the "MSC drug" that will be administered to patients for

clinical trial studies is to follow the requirement of Good Manufacturing Practice (GMP) to guarantee the safety, reproducibility, and efficiency, all aspects of the manufacturing process should be defined – e.g., approved written procedures and instructions; qualified and trained production and quality control personnel; and full traceability of MSC preparation, storage, and transportation [108].

CHAPTER 2
EXPERIMENTAL DESIGN

2.1 Cell Culture/Expansion

Human bone-marrow-derived MSCs used in this project were obtained from RoosterBio Inc. Cells were expanded once in RoosterBio expansion media in standard growth conditions (in the dark at 5% CO2 and 37C with humidity) and frozen down for the cell bank. Depending on conditions to be tested cells were either experimented on in RoosterBio media(RB media) or standard human MSC media(house media). House media is supplemented with 88.9% alpha-MEM, 10% fetal bovine serum (FBS), 1% L-glutamate, and 0.1% penicillin-streptomycin. To investigate the IFN-gamma stimulated cells, 50ng/mL IFN-gamma was added into media to stimulate the cells. For long-term expansion, media was changed 3-4days after seeding to replenish nutrients and keep the correct pH.

2.2 Bulk Cell IDO Activity Assay

The IDO activity assay was performed as previously described in Daubner et al. (1993) with some minor changes. Cells were pre-cultured 24 hours, and half of the cells were then incubated with IFN-γ for 24 hours. Next, 100uL conditioned media was collected in a round-bottom 96-well plate with 50uL of 30%(w/v) trichloroacetic acid (TCA) added to convert N-formyl-kynurenine to its delectable form L-kynurenine. Samples were then centrifuged at 600g for 5 minutes. After centrifugation, 75uL of supernatant was transferred to a flat bottom 96-well plate with 75uL of 2% (w/v) 4-(dimethylamino) benzaldehyde added in acetic acid (Ehrlich's reagent). After 5 minutes of reaction, the absorbance was read at

490nm on a plate reader. Data were normalized by media volume in culture, cell number, and day(s) in culture to get the units of pg kynurenine/cell/day. Standards were done in triplicate and in respective media using L-kynurenine supplement.

Cell number was obtained by PicoGreen quantification. Fixed adherent cells were incubated for 30 minutes in PicoGreen after being fixed with 4% (w/v) paraformaldehyde (PFA) for 15 minutes. Cells were then immediately read on a plate reader at 480nm excitation and 520nm emission as described by ThermoFisher's protocol. Serial dilution 1:2 of cells from 50,000 cells to 3,125 cells was performed to obtain the calibration curve for each donor line.

When performing the IDO activity assay over passages, the PDL was used instead of passage number to represent the age of MSCs. "Passage" refers to the transfer or subculture of cells from one flask to another after exposure to trypsin; thus, the passage number is the number of times a culture has been subcultured so that the specific number of cells present in the population is rarely considered. However, PDL is an intrinsic measure of the age of a cell line's particular culture. In addition, considering the difficulty of maintaining consistency with the passage number labeled among different laboratories, tracking with PDL is an alternative and more accurate method to represent MSC age. "PDL" refers to the total number of times the cells in the population have doubled since their primary isolation in vitro. The formula for calculating accumulate population doubling level is

$$PDL = \text{initial } PDL + 3.32 * \log(\text{total harvested cells/ total seeded cells})$$

Moreover, in order to look into the daily doubling rate of MCSs, the formula for calculating daily population doubling rate is normalized by day(s) in culture.

Considering that the plate reader has a limit of detection (LOD) and limit of quantification (LOQ), which are the lowest absorbance to be detectable or quantified respectively, LOD and LOQ were calculated and all non-detectable results were shown as ND in Figure 3.1. LOD can be calculated based on the standard deviation of the absorbance (Sy) and the slope of the standard curve (S) at levels approximating the LOD according to the formula

$$LOD = 3.3 * (Sy/S)$$

The standard deviation of the response can be determined based on the standard deviation of y-intercepts of regression lines. Similarly, LOQ can be calculated according to the formula

$$LOQ = 10 * (Sy/S)$$

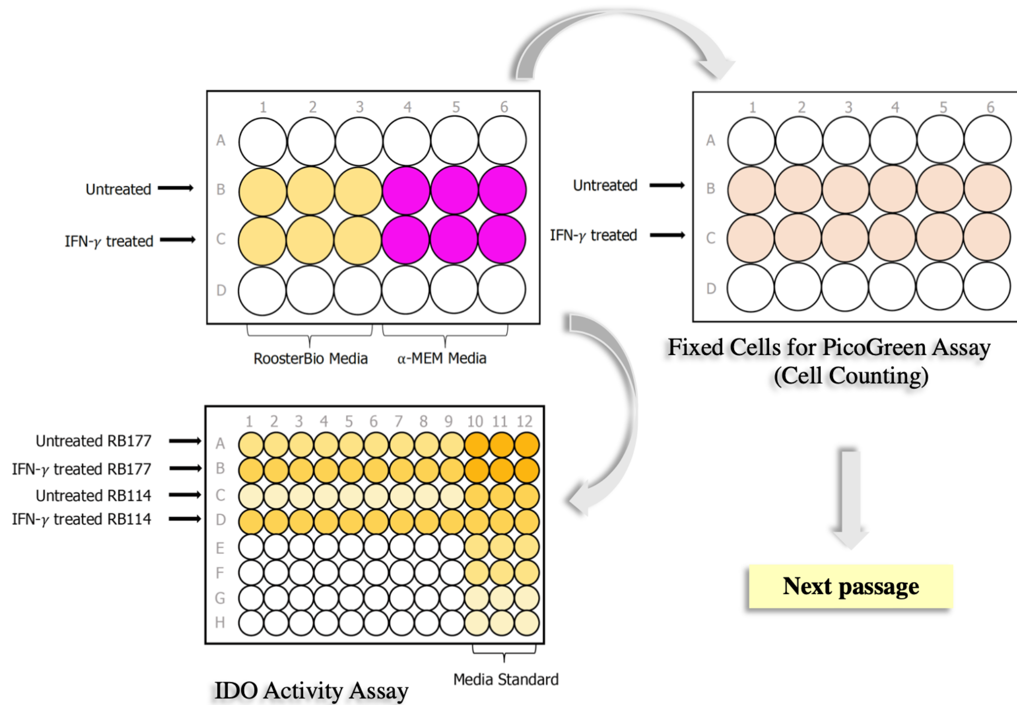


Figure 2.1: Experimental design of normalized IDO activity assay

2.3 Single cell IDO Staining Assay

2.3.1 Immunocytochemistry

MSCs thawed from the cell bank were pre-cultured for 24 hours, and half of them were then stimulated with 50ng/mL IFN-γ while the culturing media for untreated groups were changed simultaneously. MSCs were then placed in a fixative solution, which is 4%(w/v) paraformaldehyde (PFA) prepared in PBS for 15 minutes at room temperature, and then washed three times with PBS before being stained. To detect specific protein IDO, two

different IDO antibodies were used in this experiment. One is fluorescein isothiocyanate (FITC) conjugated IDO monoclonal antibody (11-9477-42) from ThermoFisher, which was only tested by flow cytometry in previous studies. Another one is unconjugated primary human IDO antibody(MAB6030) from R&D systems with secondary antibody Donkey Anti-Rabbit lgG NorthenLightsTM NL557(NL004).

1. FITC conjugated IDO Antibody

Staining using FITC-conjugated IDO monoclonal antibody(Ab) (11-9477-42) followed the manufacturer's (ThermoFisher) recommended protocol. Cells were seeded at 2500 cells/cm^2 in a clear 24-well plate. After fixation, to test intracellular protein, cells were permeabilized with 0.1% Triton X-100 prepared in PBS for 15 minutes at room temperature. Cells were washed three times with PBS and then incubated in blocking solution which is 10% bovine serum albumin(BSA) prepared in PBS for 15 minutes at room temperature. Without washing, antibodies against IDO were added and cells were incubated overnight at 4°C refrigerator. Three different concentrations of antibody at 0.12g Ab/mL, 0.24g Ab/mL, and 0.36g Ab/mL were tested in this experiment. Before fluorescence imaging, cells were washed with PBS 3 times to wash away the extra staining and decrease the background noise.

To image the stained cells fixed on a tissue culture plate, a widefield microscope in our lab was used. The LED used for excitation of FITC is 490nm (the FITC specification for 100% efficiency is 500nm; 490nm is specified as 94%). The peak emission is between 513nm-520nm.

2. Human IDO Antibody (MAB6030) with NorthenLightsTM NL557-conjugated secondary Donkey Anti-Rabbit lgG (NL004)

The staining assay used primary human IDO antibody(MAB6030) with NorthenLights^{TM} NL557-conjugated secondary donkey anti-rabbit lgG (NL004) was following the immunocytochemistry protocol suggested by R&D systems. Cells were seeded at 1500cells/cm^2 in a 4-well CultureWell^{TM} reusable gasket. After fixation, cells were permeabilized and

blocked with 0.3% Triton X-100 and 10% BSA prepared in PBS for 45 minutes at room temperature. Without washing, antibodies against IDO (20ug/mL, two times as recommended) were added, and cells were incubated overnight at $4°C$ refrigerator. Cells were then washed three times and incubated with NorthenLightsTM NL557-conjugated secondary donkey anti-rabbit lgG (NL004) (1:100, two times as recommended) for 1 hour in the dark at room temperature. As recommended, antibodies were diluted in dilution buffer consisting of 1% BSA and 0.3% Triton X-100 in PBS. Cells were washed with a wash buffer consisting of 0.1% BSA in PBS to avoid decreasing the concentration of BSA when staining. Before fluorescence imaging, cells were washed with PBS 3 times to wash away the extra staining and decrease the background noise.

As mentioned by Dae, et al. [76], cells were incubated with primary antibody against IDO for 1 hour at room temperature. We also tried to treat the cells at the same way mentioned above but shortened the incubation time of primary antibody to 1 hour as Dae described.

To image the stained cells fixed on a tissue culture plate, a widefield microscope in our lab was used. The LED used for excitation of NL557 is 565nm (specification for 100% efficiency is 557nm; 565 is 80%). The peak emission is 575nm.

2.3.2 Image Stream

Although microscopy offers detailed cellular images as well as morphological information about cells, flow cytometry is an excellent tool to obtain statistically robust results by rapidly interrogating large numbers of cells. By combining the speed and sensitivity of flow cytometry with the detailed imagery and functional insight of microscopy, the ImageStream MKII overcame the limitations of both techniques and opened the door to an extensive range of novel applications. In this experiment, CH03 (Ch width is 560 - 595nm) was used to detect NL557 conjugated human IDO antibody and CH07 (Ch width is 435 - 505nm) was used to detect DAPI(4', 6-Diamidino-2-Phenylindole).

Suspension cells prepared for flow cytometry followed the protocol recommended by

R&D systems. Cells were passaged once after thawing from the cell bank and followed by with 24 hours pre-culture. Then, after 24 hours IFN-gamma treatment, cells were trypsinized, harvested, and washed. Fixation was performed with 4% (w/v) PFA at room temperature for 10 minutes with a vortex. To detect intracellular proteins, cells were rinsed with blocking buffer at room temperature for 15 minutes with vortex. Then, suspension cells were incubated with primary human IDO antibody (MAB6030) and NorthenLights^{TM} NL557-conjugated secondary donkey anti-rabbit lgG (NL004) for 30 minutes in the dark respectively interred with one wash. Lastly, the cells were washed with permeabilization buffer one time and resuspended in the wash buffer. All the centrifuge steps after each step to decant the buffer were performed at 1500rpm for 5 minutes. Before going through the cells by Image Stream, 300nM DAPI in 0.1% Tween20 was quickly added to the suspension cells to stain the nucleus. And as described in the instructions, suspension cells stained only with DAPI or conjugated IDO antibody should be prepared for compensation.

According to the instructions, focused objects were gated based on the root mean square for image sharpness (RMS) because focused objects show a higher gradient RMS (Figure 2.2). Then, single cells were separated based on intermediate area value and a high aspect ratio (Figure 2.3). Based on focused single cells, further measurement and analysis were performed to collect and bring out the information.

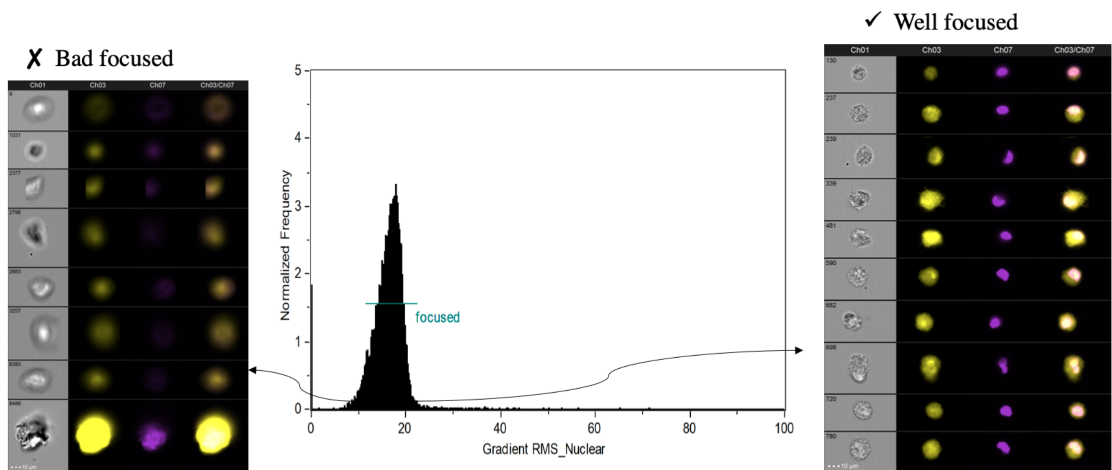


Figure 2.2: ImageStream gating strategy of focused objects
The objects with better focus have higher Gradient RMS(Root Mean Square for image sharpness) values.

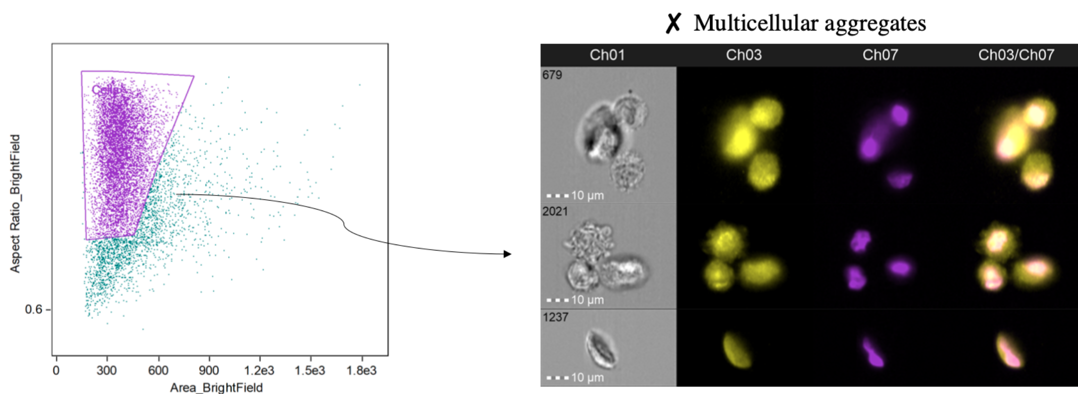


Figure 2.3: ImageStream gating strategy to select single cell events
Single cells have an intermediate Area value and a high Aspect Ratio.

2.4 Proliferation Assay

To investigate the proportion of proliferating cells in our cultures, we used Ki-67 antibody staining following the recommendations of ThermoFisher. Cells were seeded at 2500 cells/cm^2 in a clear 24-well plate. After fixation, cells were permeabilized and blocked in 0.3% TritonX-100 and 10% BSA prepared in PBS for 30 minutes at room temperature. Without washing, cells were incubated with FITC conjugated Ki-67 Monoclonal antibody(SolA15) at 10ug/mL for 1 hour at room temperature. Following this, cells were washed three times and incubated in 300nM DAPI in 0.1% Tween20 for 10 minutes at room temperature. Before fluorescence imaging, cells were washed with PBS 3 times to remove excess staining and decrease the background.

To image the stained cells fixed on a tissue culture plate, a widefield microscope in our lab was used. The LED used for excitation of FITC is 490nm (the FITC specification for 100% efficiency is 500nm; 490nm is specified as 94%). The peak emission is between 513nm-520nm. The LED used for excitation of DAPI is 365nm (the DAPI specification for 100% efficiency is 359nm; 365nm is specified as 98%). The peak emission is between 455nm-465nm.

2.5 Image Processing and Analysis

To modify the quality of fluorescence images, a Python script including a parabolic function was used to subtract the background an equalize the illumination intensity, and a Gaussian blur filter in ImageJ was used to reduce noise. Additionally, small signal noise pots were removed and single cells were segmented through ImageJ. Principle component analysis (PCA), used to demonstrate morphological features, was performed through an Rstudio script based on the features obtained with CellProfiler.

CHAPTER 3

RESULTS

3.1 IDO immunosuppressive and proliferative properties of bulk cells differs among different donors and changes over passages

Based on the normalized IDO activity assay, we set up the cells in a 24-well plate and triplicated each sample to a 96-well plate to perform the IDO activity. PicoGreen assay was performed on fixed cells to obtain the cell number. According to the detectable IDO activity normalized by cell number, comparison of IDO activity among different donors showed that cell lines with higher potency (left side in the figure) have higher normalized IDO activity and cell lines with lower potency (right side in the figure) have lower normalized IDO activity (Figure 3.1 A and B). Therefore, our normalized IDO activity assay was confirmed according to the results. In addition, although differences were still observed between reported data and measured concentration of L-kynurenine, the normalized IDO activity of IFN-γ stimulated MSCs cultured in house media is closer to the data reported by RoosterBio Company (Figure 3.1 C). Thus, the following results obtained from IDO activity assay were based on the MSCs cultured in house media.

A potential reason for the huge gap between the measured concentrations of L-kynurenine in conditioned RB media and the RoosterBio reported data may be that MSCs grow faster in RB media. As a result, there might be a reduced level of tryptophan in the media, which could provide inadequate substrate for an accurately measurement. The measured concentration of L-kynurenine in conditioned house media was close to the company's reported values, with the small difference to the reported data possibly due to additional

two cryopreservation procedures performed and expansion of the MSCs to build up our cell bank when obtained from the company which slightly lowered the immunosuppressive properties of MSCs. In addition, this can also be considered as another factor impacts the measured data in conditioned RB media.

Next, normalized IDO activity assay was performed on both high potency (RB182) and low potency (RB71) cell lines which demonstrated that an increasing tendency of normalized IDO activity from IFN-γ stimulated MSCs when they are expanded (Figure 3.1 D). One-way analysis of variance (ANOVA) demonstrated that PDL did impact the normalized IDO activity (RB182, $p = 0.001$; RB71, $p = 0.0001$). In addition, according to post-Tukey multiple-group comparison between the first and last passage during this expansion, the normalized IDO activity significantly increased at the end of the expansion (RB182, $p = 0.00004$; RB71, $p = 0.0001$). As mentioned, tracking with PDL is an alternative and more accurate method to represent the age of MSCs. With the increasing cumulative PDL, Figure 3.1 E shows a difference in daily population doubling rate over passages with a slightly decreasing trend.

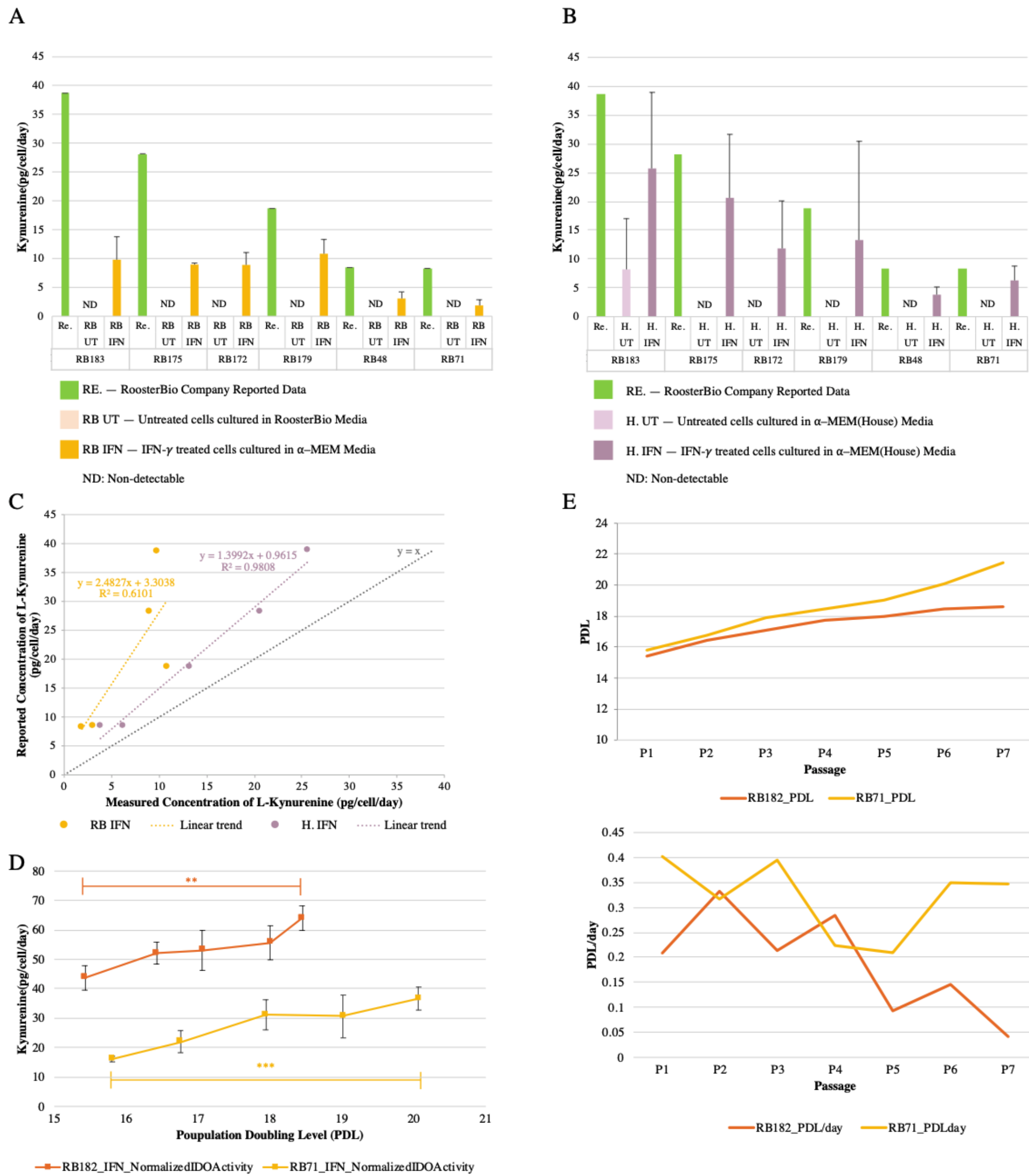


Figure 3.1: IDO activity differs among different donors and changes over passage
A: Normalized IDO activity of MSCs cultured in RoosterBio Media
B: Normalized IDO activity of MSCs cultured in standard human MSC Media (House Media)
C: Relationship between measured and company reported data
D: Normalized IDO activity of IFN-γ stimulated MSCs cultured in house media over PDL (One-way ANOVA: RB182, p = 0.001; RB71, p = 0.0001; Tukey multiple groups comparison: **p = 0.00004, ***p = 0.0001)
E: Cumulative PDL and daily population doubling rate tracking over passages

3.2 IDO immunosuppressive properties of single cell differ among different donors and change over passages

In order to explore immunosuppressive properties of a single cell, many previous studies focused on gene expression [75, 116] or protein level determined using flow cytometry [117]. In both cases, the fluorescence background is typically not a major confounding factor. However, in our work we aimed to use imaging during culture expansion to identify subpopulations of cells with high and low immune suppressive potential, which requires imaging of adherent individual cells.

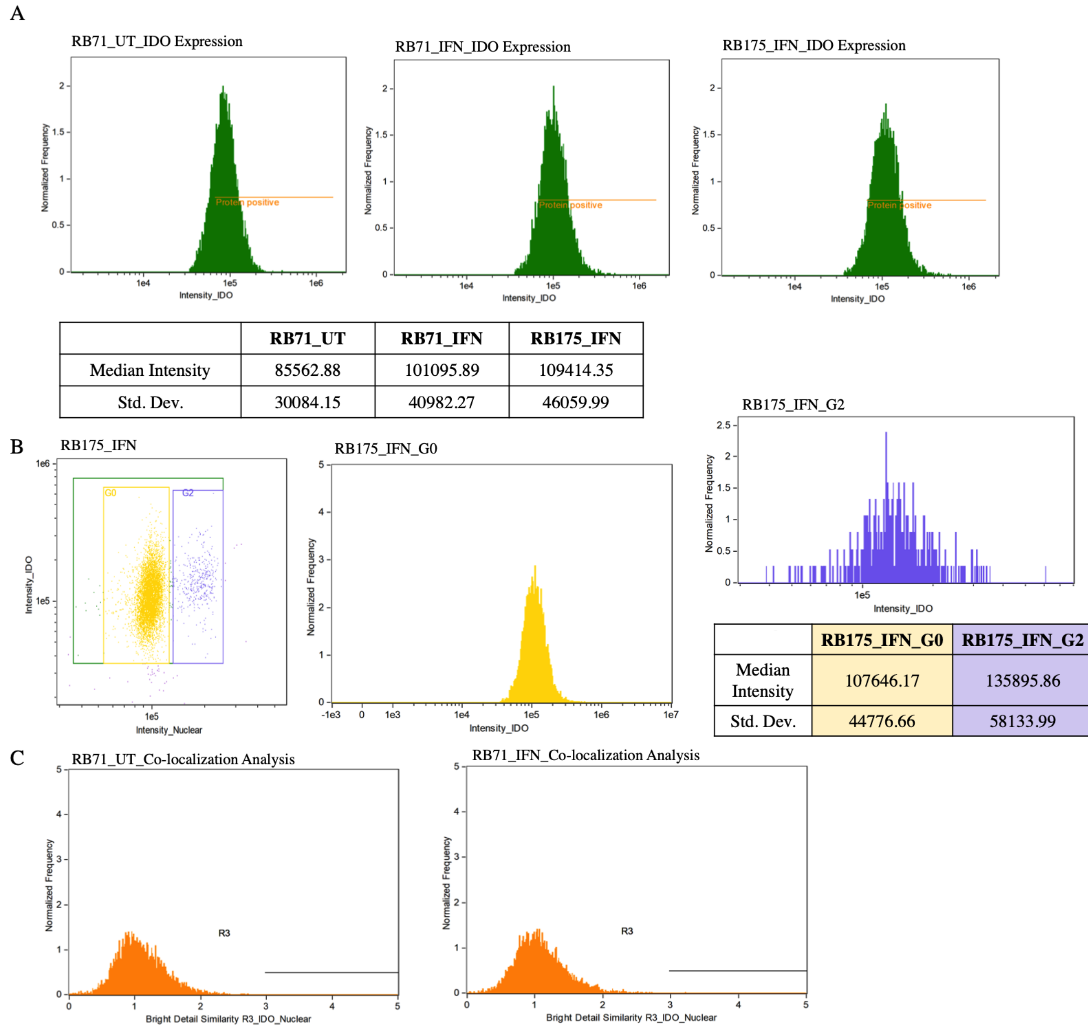
Considering the low stability of IDO in MSCs, instead of using traditional flow cytometry, Image Stream was firstly used to investigate the IDO expression at protein level as well as the relationship to nuclear expression at spatial locations. Combined with flow cytometry's excellent properties such as high speed and sensitivity, Image Stream can further provide detailed imagery and functional insight of microscopy.

Similar to results obtained with the IDO activity assay, stimulated MSCs showed higher expression of IDO at protein level and cell line with higher normalized IDO activity (RB175) showed a higher IDO expression than that of lower potency cell line (RB71) (Figure 3.2 A). The table in Figure 3.2 A represents the mean intensity and standard deviation of IDO expression. Interestingly, larger standard deviations were observed from IFN-γ treated groups as well as a wider histogram. This result suggested that a subpopulation which actively generates IDO in response to IFN-γ stimulation could exist within the MSC population.

Surprisingly, the graph (Figure 3.2 B) comparing the IDO expression and nucleus expression shows two different groups of cells whether the cells were treated with IFN-γ or not. According to the Image Stream guidance, cells gated in the left yellow box are quiescent cells at Gap1/Gap0(G1/G0) growing phases where the cells are ready to exit the cell cycle or have entered a resting state, respectively. Cells gated in the right purple box, mean-

while, are actively dividing cells at Gap2(G2) growing phase where the cells grow rapidly to prepare for mitosis. Based on the mean intensity measured by Image Stream for these two different groups, a higher IDO expression at protein level was observed from these actively dividing cells. The potential reason could be that protein was synthesized at growing phase G2 following the DNA replication at synthesis(S) phase. Therefore, the hypothesis that cell subpopulations synthesize IDO differently was further confirmed here, and an idea was inspired that it may be possible to separate highly potent MSC subpopulations according to their proliferative properties.

Because Image Stream combines the per cell information content provided by standard microscopy with the statistical significance afforded by large sample sizes common to standard flow cytometry, its best application is to locate and quantitate the distribution of signals on or within cells. A further interesting co-localization analysis was performed to investigate the IDO expression at nucleus and cytoplasm. Nuclei were stained with DAPI and IDO was stained with NL557 conjugated human IDO antibody as two probes. As described in the Image Stream instructions, the Bright Detail Similarity R3 feature is designed to specifically to compare the small bright image detail of two images and can be used to quantify the co-localization of two probes in a defined region. This is the log-transformed Pearson's correlation coefficient of the localized bright spots with a radius of 3 pixels or less within the masked area in the two input images. Figure 3.2 C demonstrates that nuclear IDO expression and cytoplasm IDO expression are uncorrelated which means they are in different spatial locations within the cells.



	RB71_UT	RB71_IFN	RB175_IFN
Median Intensity	85562.88	101095.89	109414.35
Std. Dev.	30084.15	40982.27	46059.99

	RB175_IFN_G0	RB175_IFN_G2
Median Intensity	107646.17	135895.86
Std. Dev.	44776.66	58133.99

Figure 3.2: IDO expression obtained with Image Stream (IDO stained with Nl557; nucleus stained with DAPI)
A: IDO expression at protein level show with frequency. The table below summarizes the information of mean intensity and standard deviation.
B: IDO expression at protein level of MSCs at different growing phases. (G0: quiescent cells; G2: actively dividing cells) The table below summarizes the information of mean intensity and standard deviation in relative color.
C: Co-localization analysis of IDO expression and nuclear expression of single cell.

Although Image Stream provided us convincing and conclusive information based on a large number of suspension cells, the ultimate goal of this work is to identify morphological signatures of high IDO MSCs as they are expanded in vitro. Thus, we following tried the more challenging strategy – adherent cell immunocytochemistry to collect information about IDO expression as well as morphological features of adherent individual cells.

However, due to the low stability of IDO expression in MSCs, IDO staining was dim and the detected signal was obscured by strong background. To address this problem, despite a very long exposure time (up to 20 seconds) to strengthen the input signal images were noisy with low signal-to-background ratios. To obtain analyzable and quantitative information from these images, pre-processing and experimental optimization were performed (Figure 3.3 A) to recover image features and investigate and analyze the segmented single cells. Although image quality was poor, cell segmentation was still possible in most cases. The range of mean intensity of segmented single cells was plotted 300 to 900 with a 100 step (Figure 3.3 B left), and an obvious difference between untreated and IFN-γ treated group was observed. This suggested that immune stimulation by IFN-γ, which we found to increase IDO activity, also increased the amount of IDO enzyme present in the cells. In addition, the histogram provided not only a visualized comparison between two groups but also information that subpopulations with different levels of IDO may exist in a single MSC culture due to the span of cell frequencies across the IDO intensity range. Therefore, a scatter plot of each single cell (Figure 3.3 right) further confirmed our hypothesis and showed a difference in cell area. According to these results, we hypothesized that subpopulation might exist in the same group of MSCs that express IDO at protein level differently, and cell area could be a potential parameter to identify these subpopulations.

Although image quality has been modified through a couple of steps, the input signal was still low due to the IDO instability as well as the highlighted effect of background, which makes the experiment time- and cost-extensive. A secondary antibody strategy was then applied to improve the signal-to-background ratio, which had the advantage of

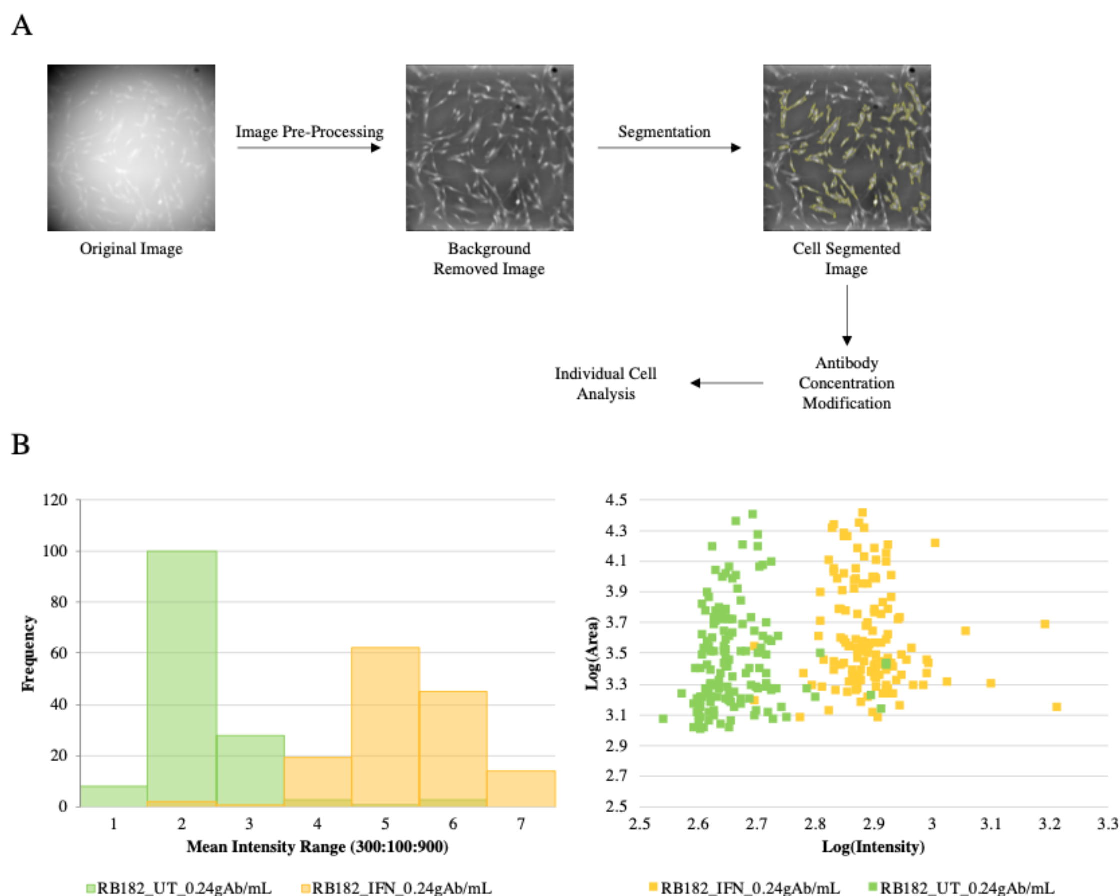


Figure 3.3: Immunofluorescence staining of FITC conjugated IDO antibody
A: Pre-processing to obtain individual cell information
B: Immunofluorescence staining of FITC conjugated IDO antibody (11-9477-42) from untreated (UT) and IFN-γ treated (IFN) MSCs at a concentration of 0.24g Ab/mL

a brighter fluorescent secondary antibody in a lower background spectral region that could enhance the signal-to-background ratio with a shortened exposure time. The images shown in Figure 3.4 A are the samples where MSCs have performed immunofluorescence staining of human IDO antibody attached with NL557 pre-conjugated secondary antibody from MSCs with low potency (RB71) and high potency (RB175) at low passage (P3) and high passage (P6). Similarly, MSCs stimulated with IFN-γ show a higher IDO expression than that of untreated MSCs which confirmed the results obtained from normalized IDO activity assay in our lab (Figure 3.4 B). We compared the difference of untreated and IFN-γ treated

groups from each condition. According to the difference between IFN-γ treated groups to the relative control group (untreated group), MSCs with high potency (RB175) showed a greater increase in subtracted mean intensity than MSCs with low potency (RB71) after stimulating with IFN-γ. In addition, stimulated MSCs at high passage (P6) displayed a larger relative enhancement in the subtracted mean intensity. Due to the lack of an untreated group of high-potency MSCs (RB175) at high passage (P6), no conclusive information can be provided here about the comparison between MSCs with low and high potency at high passage.

Since these studies are in process, more experiments and subpopulation categorizations are needed to bring out convincing, conclusive information. However, the image pre-processing and experimental optimization completed here enable further studies on segmented individual cells. Additionally, as expected, the collection of morphological features, as well as analysis based on morphological features, will also offer the changing over passage from MSCs with high and low potency.

In summary, the results obtained from suspension cells with Image Strean and adherent cell imaging further confirm our results obtained from normalized IDO activity assay and the hypothesis about existing subpopulations in the same group of MSCs. Furthermore, identification of subpopulations based on proliferative properties and cell morphological features is considered as a novel and feasible way to identify the high potency MSCs during in vitro expansion.

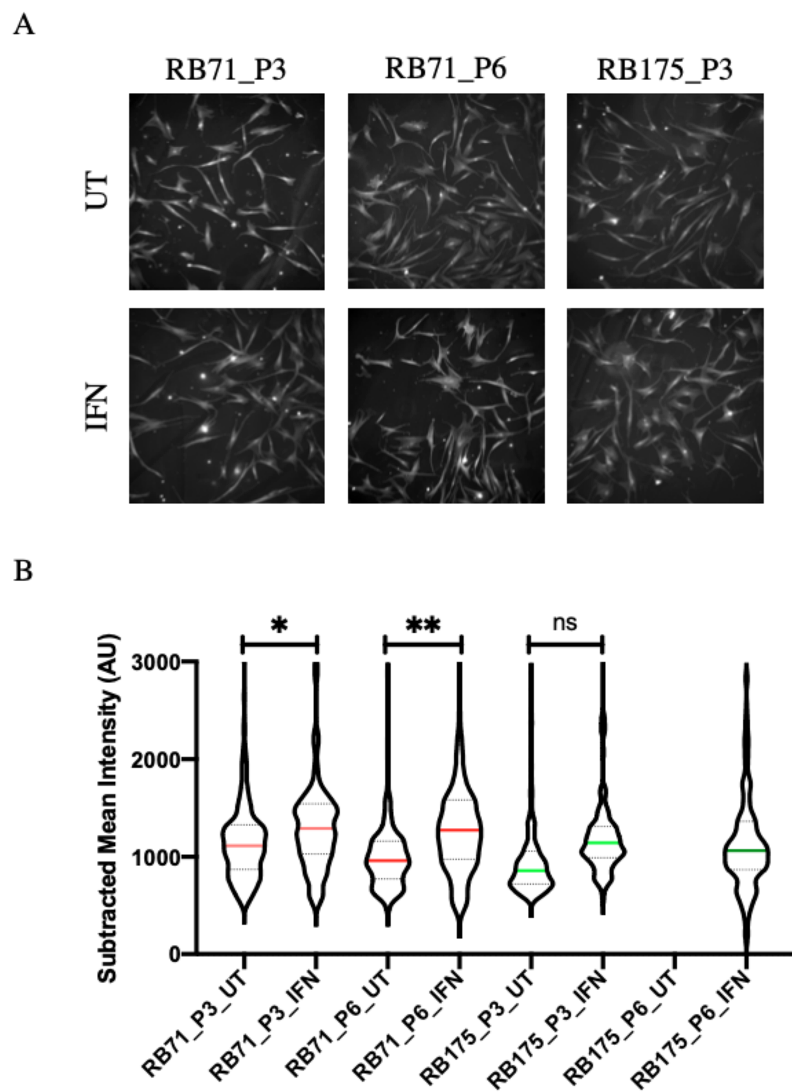


Figure 3.4: Immunofluorescence staining of human IDO antibody (MAB6030)
A: Immunofluorescence staining of human IDO antibody (MAB6030) from untreated (UT) and IFN-γ treated (IFN) MSCs at low passge (P3) and high passage (P6) with low potency (RB71) and high potency(RB175).
B: Analysis of mean intensity subtracted with background between different conditions

3.3 Percentage of proliferating MSCs and nuclear features differ among different culture conditions

Since fewer cells were always obtained from IFN-γ groups than cells obtained from untreated groups when performing the immunocytochemistry experiment, we wondered whether IFN-γ treatment affected the MSC proliferation or not. Previous studies have indicated that IFN-γ-induced IDO activation in MSCs leads to impaired proliferation and an alteration of their differentiation capacity [78]. Therefore, we performed proliferation assay through Ki-67 monoclonal antibody to investigate the percentage of actively proliferating MSCs from untreated and IFN-γ treated groups. Ki-67+ cells are in G1, S, G2, and M growing phases where the cells are highly dividing and proliferating. Surprisingly, although the number of proliferating MSCs in untreated and IFN-γ treated group was not significantly different, principle component analysis (PCA) indicated that the nuclear features shifted after IFN-γ stimulation. The results here explain the majority of the variance in the data set (88.61%) with PC1 explaining 56.18% and PC2 explaining 32.43% of the variance in the data (Figure 3.5 C). The parameters in the Appendix 1 that are impacting the nuclear features offered further detailed information that negative loadings indicate a decrease and positive loadings indicate an increase.The top five parameters find a decrease in form factor and increase in eccentricity, indicating that the nuclear became more elliptical after IFN-γ stimulation. In addition, the decrease in extent or solidity, as well as the increase in compactness, suggested that the nuclear stimulated with IFN-γ shows a more irregular boundary than untreated ones. The results here suggest that nuclear features are a potential predictor to identify different subpopulation even if small differences were observed from the bulk cell experiment.

Additionally, due to a decreased tendency of daily population doubling rate observed in normalized IDO activity assay (Figure 3.1 E), we hypothesized that the percentage of proliferating MSCs lowered when they are expanded. Therefore, a proliferation assay was

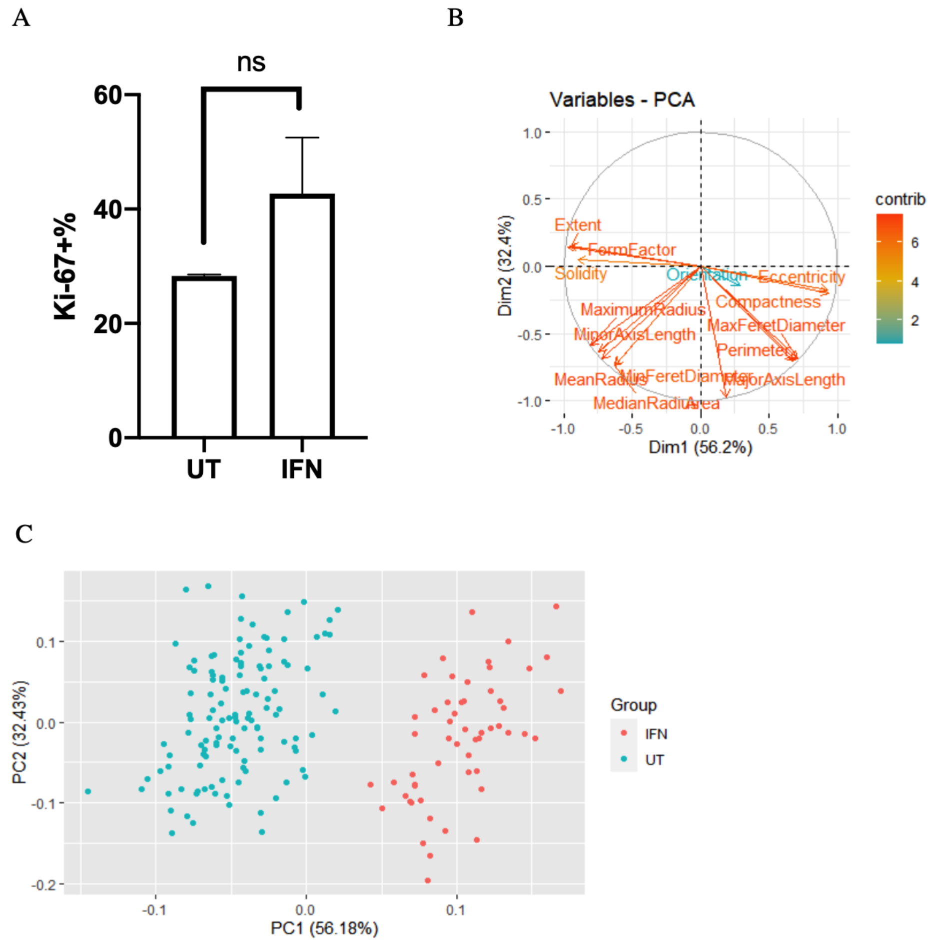


Figure 3.5: IFN-γ stimulation shifts percentage of proliferating MSCs and nuclear features
A: Percent of Ki-67+ MSCs from untreated (UT) and IFN-γ treated (IFN) group (ns = no significant difference)
B-C: Principle component analysis (PCA) and correlation circle of nuclear features obtained with CellProfiler

also performed to detect the percentage of proliferating MSCs in the MSC population (Figure 3.6 A). A comparison between low passage (P1) and high passage (P5) based on the percentage of Ki-67+ MSCs shows that the amount of proliferating MSCs significantly reduced at high passage (Figure 3.6 B). With this significant difference, we also wondered about alterations in MSC nuclear features of MSCs in different conditions. As shown in the results obtained with PCA, proliferating cells showed an obviously right shift along with PC1 (56.58%) from all of the cells based on the nuclear features (Figure 3.6C). According to the top five parameters that impact the nuclear features in Appendix 2, we can conclude

that proliferating cells are showing a larger nuclear size than all of the cells, snd this is consistent with the characteristics of cells at interphase in the cell cycle.

Moreover, MSCs represented different nuclear features at low passage (P1) and high passage (P5) along with PC2 (22.61%)(Figure 3.6D). Similarly, the PC2 loading in Appendix 3 shows that solidity increased and extent decreased, which indicates that the nuclei of aged MSCs at passage 5 have a more regular boundary than those of fresh MSCs at passage 1. Furthermore, a decrease in form factor and minor axis length suggested that the morphology of nucleus becomes more elliptical and flatter when MSCs are expanded in vitro.

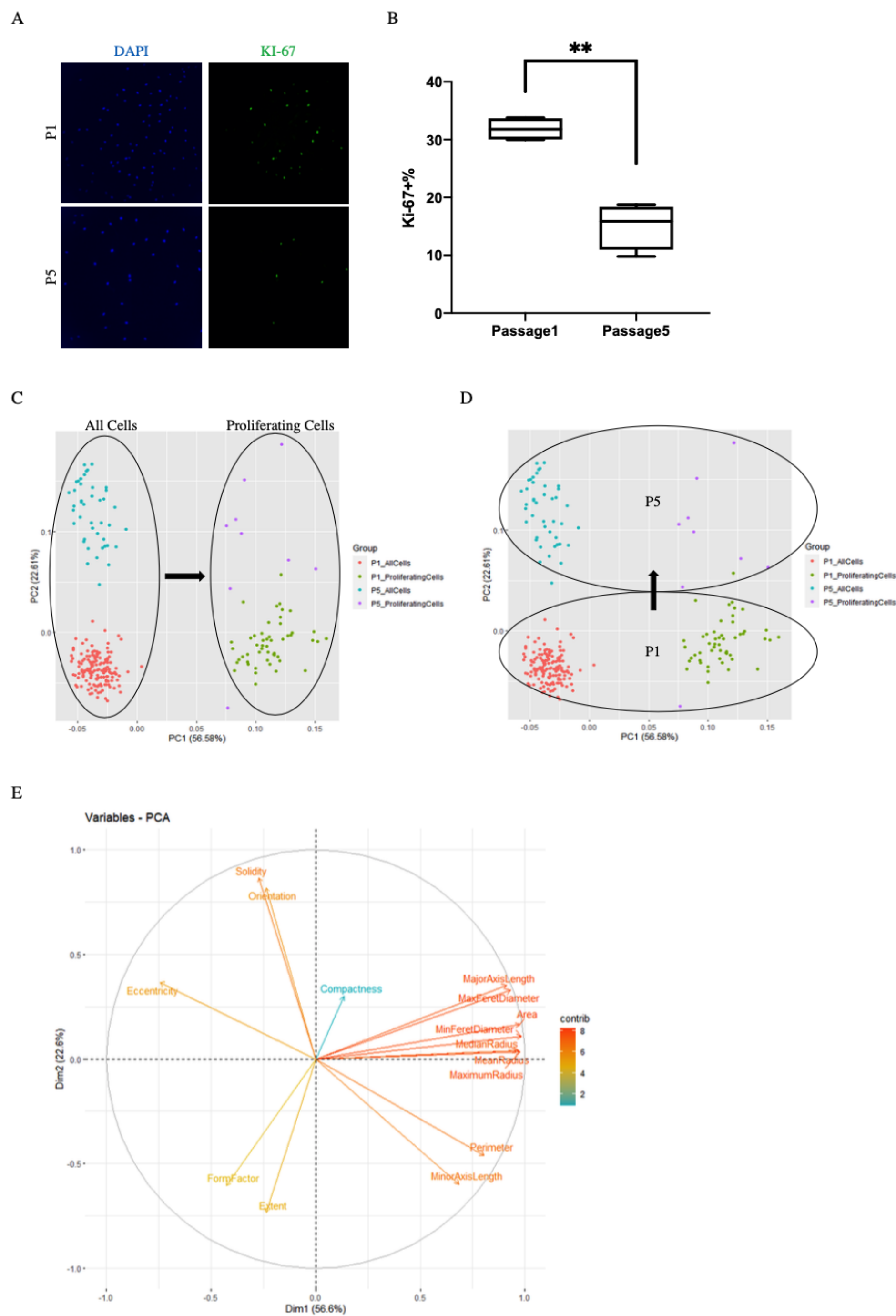


Figure 3.6: Percentage of proliferating MSCs and nuclear features differs over passages
A: Immunofluorescence staining of DAPI (blue) and Ki-67(green) from MSCs
B: Percent of Ki-67+ MSCs from low passage (P1) and high passage (P5) (**p = 0.0025)
C-E: Principle component analysis (PCA) and correlation circle of nuclear features obtained with CellProfiler

CHAPTER 4

CONCLUSION

According to phase I clinical trials [118], MSCs showed no toxicity to patients at concentrations from 1 to 50 million cells/kg based on the patient's body weight. Because of the large amount required for clinical treatment, MSC culture expansion in vitro with cryopreservation is critical. However, many previous studies have demonstrated that immunosuppressive properties, as well as MSC morphological features, changed when they are expanded [114, 119]. Therefore, this project's main goal is to explore and understand the changes in MSCs immunosuppressive and proliferative properties over passages in combination with MSCs morphological features.

In this project, according to the normalized IDO activity assay based on bulk cells and investigation of IDO expression at protein level based on a single cell, IFN-γ stimulation of IDO activity and expression was observed (Figure 4.1 B, C, D and E). In addition, IDO expression and normalized IDO activity consistently showed the different potency of mesenchymal stem cell lines (Figure 4.1 A, C, E and F). Interestingly, although it was not expected, both normalized IDO activity and IDO expression showed a consistent increasing tendency over passage during in vitro culture expansion (Figure 4.1 A and C). However, when combined with the gap between the measured and reported normalized IDO activity (Figure 3.1 A, B), the increasing trend can be possibly understood as the recovery of MSCs during culturing expansion after thawing. In addition, although a slight decreasing trend was observed from the daily population doubling rate, the cumulative PDL kept increasing during the expansion in this project which indicated that the cultured MSCs were still dividing at the end of this expansion instead of becoming senescent (Figure 3.1 E). There-

fore, we believe that normalized IDO activity may reach a stable state at the end of in vitro expansion where cells reach senescence or there is no change in PDL from one subculture to the next.

According to the statistically robust information obtained with Imagestream, we observed the stimulation of IDO expression as well as the difference between cell lines with high and low potency (Figure 4.1 D-F). Although higher nuclear IDO expression was observed in immunofluorescence images, co-localization analysis performed based on IDO expression and nuclear expression showed there is low correlation of cytoplasm IDO expression to nuclear IDO expression at the spatial locations. This also partially provided us some information about IDO distribution in MSCs. According to the difference of IDO expression in the same group of MSCs was observed both in immunocytochemistry (Figure 4.1 B) and ImageStream (Figure 4.1 D-F), we hypothesized that a subpopulation exists that expresses IDO at protein level differently. Further evidence obtained with ImageStream showed that IDO expression of quiescent MSCs is lower than that of actively dividing MSCs (Figure 4.1 G-I). Therefore, we tried to understand cellular characteristics, protein expression, and nuclear features at different growing phases of cell cycle in-depth. The eukaryotic cell cycle consists of four distinct phases: Gap1(G1), Synthesis(S), Gap2(G2), and Mitosis and cytokinesis(M). Cells that have temporarily or reversibly stopped dividing will enter a state of quiescence called Gap0(G0) phase. Generally, cells increase in size in G1 and DNA replication occurs during the S phase. Therefore, higher IDO expression was observed from MSCs at G2 (Figure 4.1 G-I). In combination with the proliferation assay through Ki-67 monoclonal antibody, further information showed that MSCs had a lower percentage of proliferating cells at high passage during in vitro culture expansion which indicated a loss of proliferative activity when MSCs are expanded.

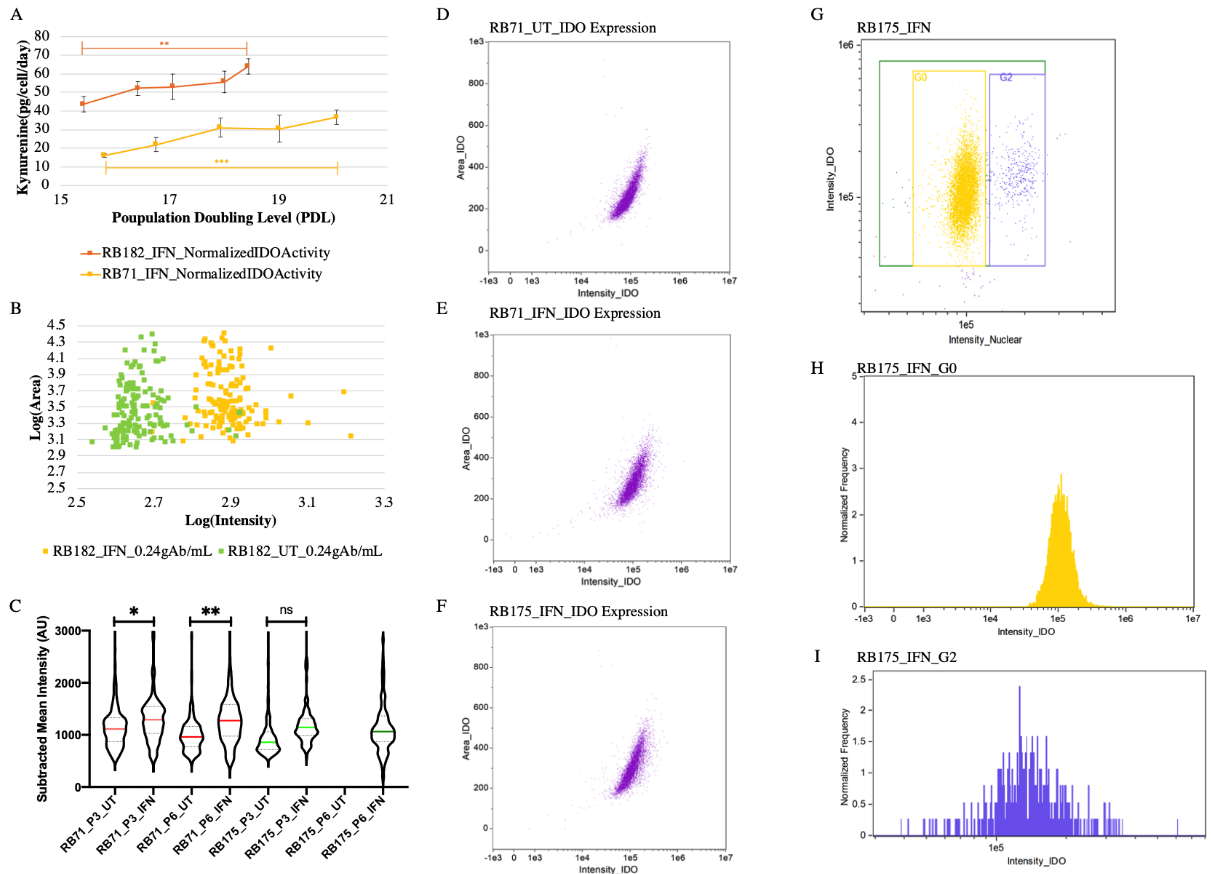


Figure 4.1: Summary figure of IDO assays
A: Normalized IDO activity of IFN-γ stimulated MSCs cultured in house media over PDL (One-way ANOVA: RB182, $p = 0.001$; RB71, $p = 0.0001$; Tukey multiple groups comparison: **$p = 0.00004$, ***$p = 0.0001$) .
B: Immunofluorescence staining of FITC conjugated IDO antibody (11-9477-42) from untreated (UT) and IFN-γ treated (IFN) MSCs at a concentration of 0.24g Ab/mL.
C: Immunofluorescence staining of primary human IDO antibody(MAB6030) with NorthenLightsTMNL557-conjugated secondary donkey anti-rabbit lgG (NL004).
D-F: IDO expression at protein level obtained with ImageStream showing with IDO area.
G-I: IDO expression at protein level of MSCs obtained with ImageStream at different growing phases. (G0: quiescentcells; G2: actively dividing cells)

At the end of this project, we used principle component analysis (PCA) to analyze the nuclear features. According to the results, although the percentage of proliferating cells was not significantly different from untreated and IFN-γ treated groups, nuclear features obtained with PCA were indeed impacted by IFN-γ stimulation. Moreover, proliferating MSCs at low and high passage can be identified based on nuclear features. This part of the project offers an opportunity to screen actively dividing MSCs with high IDO potency rapidly based on their morphological features during large-scale culture expansion.

CHAPTER 5
DISCUSSION AND FUTURE WORK

Our work at this point in the project successfully provided us information on immunosuppressive and proliferative MSC properties. This offers an opportunity for later identification and separation of highly dividing MSC subpopulations with high immune potency during culture expansion. Although this is a good start to identifying the immunosuppressive and proliferative properties for desirable MSCs, more work should be performed on morphological-feature measurement and analysis so that we can realize a rapid identification and separation of desirable cells during large-scale expansion.

In the future, firstly, morphological features and further analysis based on sufficient amounts of MSCs should be performed to provide convincing information for identifying different subpopulations; this will be based on the pre-processing and experimental optimization have already been completed in the project to get analyzable and quantitative information from immunofluorescence images stained with human IDO antibody. Furthermore, according to the collected morphological features, high potency and low potency MSCs could be identified before culturing expansion.

In addition, further experiments are required for normalized IDO activity assay. A long-term expansion with higher cumulative PDL could provide more convinced and reliable information about IDO activity as we believe that normalized IDO activity may reach a stable state at the end of in vitro expansion where cells reach senescence or there is no change in PDL from one subculture to the next. In combination with the findings obtained from proliferation assay, senescence markers can also be considered as a potential indicator to represent MSC condition and age during culture expansion. Usually, senescence-associated

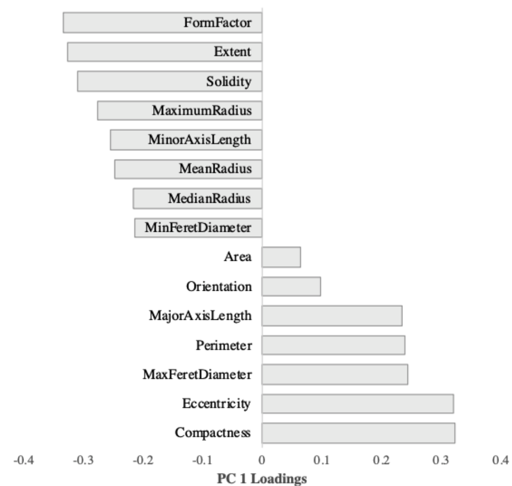
beta-galactosidase (SA-β-Gal) assay is the most commonly used method to investigate the role and changes of senescence markers during in vitro culture expansion. However, a recent study published in 2019 proposed the use of endogenous autofluorescence as real-time quantification of cellular senescence in human MSCs, based on label-free flow cytometry analysis [81]. Using endogenous autofluorescence to quantify cellular senescence also offers us a chance to perform label-free analysis on MSCs with doing less harm on them.

In the end, immunosuppressive and proliferative properties obtained here can be combined with MSC morphological and senescent properties to predict and identify MSC subpopulation with high immune potency and excellent growing conditions during in vitro large-scale culture expansion.

APPENDIX

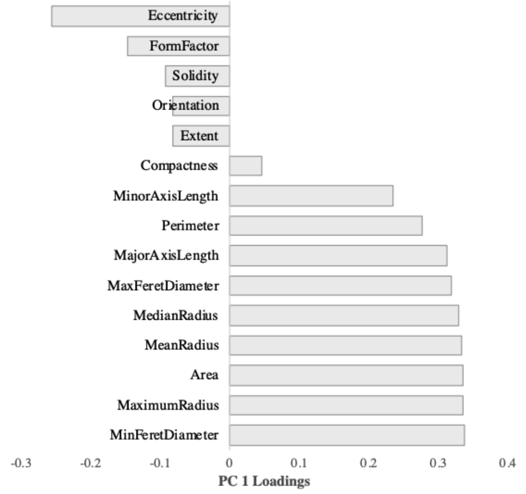
Appendix 1: Supplement for Figure 3.5

Parameters	PC1 Loadings
Area	0.06499882
Compactness	0.32237429
Eccentricity	0.32003382
Extent	-0.3271858
FormFactor	-0.3341398
MajorAxisLength	0.23356373
MaxFeretDiameter	0.24345651
MaximumRadius	-0.2758708
MeanRadius	-0.2462895
MedianRadius	-0.2168162
MinFeretDiameter	-0.2130366
MinorAxisLength	-0.255691
Orientation	0.09876601
Perimeter	0.23960097
Solidity	-0.3085562



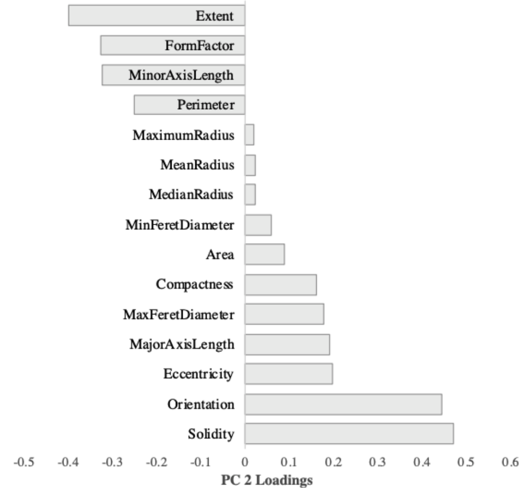
Appendix 2: Supplement for Figure 3.6 C

Parameters	PC1 Loadings
Area	0.33483154
Compactness	0.04608457
Eccentricity	-0.2552802
Extent	-0.0817707
FormFactor	-0.1459905
MajorAxisLength	0.31314268
MaxFeretDiameter	0.31973354
MaximumRadius	0.33562374
MeanRadius	0.33251666
MedianRadius	0.32886455
MinFeretDiameter	0.33743018
MinorAxisLength	0.23536744
Orientation	-0.0818129
Perimeter	0.2762402
Solidity	-0.0932045



Appendix 3: Supplement for Figure 3.6 D

Parameters	PC2 Loadings
Area	0.08921001
Compactness	0.16271647
Eccentricity	0.19851764
Extent	-0.3989865
FormFactor	-0.3277788
MajorAxisLength	0.19159046
MaxFeretDiameter	0.1791623
MaximumRadius	0.01903995
MeanRadius	0.02202735
MedianRadius	0.02243849
MinFeretDiameter	0.05908915
MinorAxisLength	-0.3246577
Orientation	0.44430432
Perimeter	-0.249489
Solidity	0.46991917



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