

Towards A Serum-Free Medium: Growth Receptors And Signaling Pathways That Regulate Multipotency In Human Mesenchymal Stem Cells

Nicholas Xue Wei Tan, Felicia Soo Lee Ng, and Vivek Tanavde

Abstract—Human mesenchymal stem cells (MSCs) are capable of being used in human therapeutic procedures such as tissue repair and gene therapy due to their multipotency. However, the expansion of MSCs is limited by the restricted use of fetal bovine serum (FBS) as the nutritional supplement. Hence, contamination by pathogens from the xenogenic component can be eliminated if a serum-free media were used.

In this paper, we identified the human growth receptors (HGRs) and corresponding signaling pathways expressed in undifferentiated proliferative MSCs by analyzing available microarray data. The microarray datasets were generated by Affymetrix GeneChips and Illumina BeadArrays. The MSC samples used were cultured in both fetal bovine serum (FBS) and autologous serum (AS) at passage one. Results from the different platforms indicated 94 similarly expressed human growth receptors. Affymetrix was also able to detect 129.19% more HGRs than Illumina. We also discovered that HGR expressions across different types of serum were similar, indicating that type of serum had little impact on proliferation mechanisms in early passages. Next, Ingenuity Pathway Analysis identified 11 signaling pathways that were expressed in all four MSC cultures. In particular, the Notch Signaling Pathway and Transforming Growth Factor – Beta Signaling Pathway were consistently expressed in all MSC cultures. These 11 signaling pathways and 94 HGRs are important in understanding the maintenance of MSC undifferentiated proliferative state, and the corresponding growth factors are possibly required minimally in developing a serum-free medium for MSC expansion.

Index Terms—Mesenchymal stem cells, microarray, signaling pathway, growth factors, growth receptors

I. INTRODUCTION

Human mesenchymal stem cells (MSC) are most commonly isolated from bone marrow and are expanded in vitro easily. Attributable to their multipotency, MSCs harbour much potential to be used in various human therapeutic procedures. They are capable of differentiating into bone [1,2], cartilage [3], fat [4], and a few other tissue types [5, 6, 7, 8], hence they might be key in treating genetic disorders [9]. U. S. Food and

Drug Administration approved the first clinical application of MSC in children with osteogenesis imperfecta [10], and a few other therapeutic applications are currently undergoing trials, for example metachromatic leukodystrophy [19] and breast cancer [20]. While most research is centered on MSC cell fate determination – the differentiation of MSCs into various tissue lineages, there has been hardly any research into the mechanisms that regulate MSC self-renewal and proliferation.

Although MSCs are multipotent and the tissues that they can regenerate compares poorly with the pluripotency of embryonic stem cells (ESC) [15], MSCs do have some advantage over ESCs. MSCs are obtained from an initial culture of the patient's own cells for expansion. Hence, most ethical and legal issues pertaining to sourcing are avoided. In addition, since MSCs are multipotent, directing them to a desired fate is less challenging compared to embryonic stem cells [9].

Because MSCs are present in spare amounts in the bone marrow, expansion is necessary before performing research and clinical tests [12]. In this aspect, the expansion and culture of MSCs is limited, technically and with respect to clinical potential, by the restricted use of fetal bovine serum (FBS) as nutritional supplement [15]. Some studies have reported success with autologous serum (AS) [13, 16] as an alternative for MSC culture. However, all current techniques of MSC expansion in vitro still contain in some part animal serum because the growth factors that are minimally required to fully support the growth of MSCs are still unknown [11].

The therapeutic potential of MSCs lies in the transplantation of differentiated cell types [9, 17], thus it is vital to eliminate pathogenic contamination from the xenogenic component and allow large-scale culture. Current serum media restricts human therapeutic transplantations due to xenogenic contaminants, large variability in MSCs from lot to lot which results in variable experimental results, increased work load and limited large-scale culture due to resource-intensive serum production. One in six patients with osteogenesis imperfecta who received expanded MSCs demonstrated an immunological anti-FBS response [10]. Therefore the development of a serum-free medium for MSC expansion will provide reproducible culture conditions, allow large scale expansions, and also enhance the

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Nicholas Xue Wei Tan was with Raffles Junior College.the Bioinformatics Institute (e-mail: nicktxw@yahoo.com).
Felicia Soo Lee Ng and Vivek Tanavde are with Bioinformatics Institute.

clinical potential of MSCs in treatment [14].

In this study, we explore the development of a serum-free medium by identifying the signaling pathways and human growth receptors (HGRs) that regulate proliferation and maintain multilineage differentiation potential in MSCs. Microarray analysis was performed on available microarray datasets of undifferentiated MSCs cultured in FBS and AS at passage 1. The microarray datasets were generated using Affymetrix GeneChips and Illumina BeadArrays. Results from the different serum conditions and microarray platforms were analyzed and consolidated. HGR expression was compared across different microarray platforms and types of serum because the signaling pathways regulating MSC self-renewal and maintenance of multipotency are universally defined. All expressed HGRs and their corresponding signaling pathways were identified. A total of 11 signaling pathways and 94 common HGRs were expressed in undifferentiated proliferative MSCs. The Notch Signaling Pathway and Transforming Growth Factor-Beta (TGF- β) Signaling Pathway were reported to be consistently expressed in undifferentiated proliferative MSCs by analysis using Ingenuity Pathway Analysis (Ingenuity® Systems, www.ingenuity.com). These signaling pathways and their corresponding growth factors may be valuable for developing methods to maintain and propagate MSCs in a serum-free medium as well as to regulate their differentiation into different mesenchymal lineages.

II. MATERIALS AND METHODS

A. MSC culture and Microarray Data

In this study, microarray data from four MSC samples were used. The Affymetrix GeneChips datasets which consist of one MSC sample cultured in FBS and one MSC sample cultured in AS were obtained from the public repository ArrayExpress (<http://www.ebi.ac.uk/arrayexpress/>). These microarray assays were performed by Shahdadfar et al. [12]. The unpublished Illumina BeadArray microarray assays of the other two MSC samples cultured in FBS were performed by Invitrogen Corporation.

For the microarray assays performed by Affymetrix GeneChips, cRNA from the samples were hybridized to the HG-U133A array containing 22,284 probes, which corresponds to roughly 14,500 genes. Agilent Gene Array Scanner (Affymetrix) was used to scan the arrays. The resultant gene expression data was not processed using manufacturer software.

Similarly, the gene expression data from the Illumina BeadArray platform was not processed using manufacturer software.

B. Human growth receptors (HGRs) expression

Data processing was conducted by Microsoft Excel. The raw intensity of individual samples was normalized and

scaled. The intensity-based log ratio median method was employed for data normalization across the four gene expression datasets. Genes from the datasets met the signal intensity cut-off of fifty were considered to be expressed and used in analysis.

All known HGRs that were expressed in each individual microarray analysis were identified. HGR expression was compared between microarray platforms, as well as between types of serum. Although the two microarray platforms did not produce entirely similar results due to their different microarray technologies, the gene expression data from the two platforms were comparable especially after normalization.

Finally, HGRs that were expressed across all four microarray analyses were identified.

C. Ingenuity Pathway Analysis

Pathway analysis was performed by Ingenuity Pathway Analysis.

The HGRs that were considered to be expressed across all four microarray analyses were input into Ingenuity Pathway Analysis. The canonical pathways in the Ingenuity Pathways Knowledge Base that were associated with these expressed HGRs were identified. Out of the 11 common signaling pathways reported, we decided to focus on the overall expression of Notch Signaling Pathway and TGF-beta Signaling Pathway across MSC datasets because there were slightly more HGRs expressed in those pathways.

III. RESULTS

The HGR expression profiles of the four MSC cultures were reviewed using established procedures for Affymetrix GeneChips and Illumina BeadArrays.

A. Type of serum does not affect HGR expression in Passage 1

Microarray analyses of the two MSC samples expanded in FBS and AS at passage one were performed by Affymetrix GeneChips. The normalized microarray data showed almost identical HGR expression despite the different culture mediums. The MSC sample cultured in FBS expressed 310 HGRs compared to 318 HGRs expressed in the MSC sample cultured in AS. The MSC sample cultured in AS expressed 2.58% more HGRs than MSCs cultured in FBS. The reported difference is quite insignificant and might be attributed to random error in the microarray assay. In addition, 308 HGRs were similar between the two MSC samples. Hence, there is high correlation of HGR expression between different culture mediums (Table I) in this early passage.

Table I: HGRs expression across different microarray platforms and serum types

	Number of expressed HGRs	Number of similar HGRs expressed across each platform
Affymetrix FBS	310	308
Affymetrix AS	318	
Illumina FBS 1	138	124
Illumina FBS 2	136	
Common HGRs across all analyses	94	-

B. Affymetrix GeneChips detected 129.19% more HGRs than Illumina BeadArrays

Affymetrix platform reported 310 and 318 expressed HGRs in MSC samples cultured in FBS and AS respectively. Illumina platform reported 138 and 136 expressed HGRs in the MSC samples cultured in FBS. On average, Affymetrix detected 314 HGRs compared to Illumina that detected 137 HGRs. Hence, Affymetrix detected 129.19% more HGRs than Illumina.

C. Common HGR expression

A total of 94 human growth receptors met the signal intensity cut-off of fifty consistently across the all four microarray analyses (Table II).

D. Signaling Pathways

The 94 expressed HGRs were input into Ingenuity Pathways Analysis. Canonical pathways that were associated with at least 1 of the 94 HGRs in Ingenuity Pathways Knowledge Base were identified. Table III shows the 11 signaling pathways that consisted of at least one of the above 94 human growth receptors. The signaling pathways reported were limited to Ingenuity Pathways Knowledge Base. These signaling pathways and their corresponding growth factors are important in understanding how MSCs maintain and proliferate as well as in developing a serum-free medium for expansion.

E. Notch Signaling Pathway and TGF-β Signaling Pathway Overall Expression

Ingenuity Pathway Analysis was used to determine the

expression of the Notch Signaling Pathway and TGF-β Signaling Pathway across each of the four global gene expression datasets. Both pathways were consistently expressed across all four microarray analyses. Fig. 1 shows the overall expression of the Notch Signaling Pathway in the AS culture with Affymetrix as the microarray platform. Fig. 2 shows the overall expression of the TGF-β Signaling Pathway in the FBS culture with Illumina as the microarray platform. The figures show all objects associated in that particular pathway. The shade of each object represents the expression intensity. Higher levels of expression are represented by darker intensities.

The Notch Signaling pathway is commonly involved in intercellular signaling, proliferation, and cell fate determination during development. Ligands of the Notch Signaling Pathways belong to two subclasses of the Delta and Serrate-Lag2 family. In the human, there are two members from the Serrate family (JAG1 and JAG2) and three members from the Delta family (DLL1, DLL3 and DLL4). These ligands can bind with four Notch receptors (NOTCH1, NOTCH2, NOTCH3, NOTCH4). From Fig. 1, it can be seen that only 3 ligands were expressed (JAG1, JAG2, DLL3) and all four Notch receptors were expressed.

Table II: HGRs expressed across all microarray platforms and serum types

BZRP	NPR3	FZD7	IL10RB	TNFSF5IP1	IGFBP2	FZD6	P2RY11
NPTN	TNFRSF25	C11orf75	ADIPOR2	CLEC11A	NOTCH2	BMPR1A	FZD5
RAC1	TMED1	LPHN2	RARA	ADIPOR1	GHR	PSEN1	ROR1
AXL	OSMR	TRAF5	GRINL1A	FZD2	OGFR	ACVR2A	P2RY5
PDGFRB	PTPRN	LGR4	FZD1	GPR1	FZD4	RXR	PTPRA
CRLF1	ACVR1B	IGF2R	IL4R	IL13RA1	NOTCH3	GABBR1	BDKRB1
TNFRSF1A	NPR2	GPR124	PGRMC1	TMEM147	GFRA1	IL17RC	PILRB
PGRMC2	RAMP1	GPR172A	IL1R1	TNFRSF10B	GRM4	CMKOR1	FZD9
IFNGR2	NOTCH1	GPR175	ADORA1	RXRA	CSF1R	CRLF3	ACVR1
OPRS1	TNFRSF6B	PTPRG	NLE1	TNFRSF11B	FAS	BMPR2	TNFRSF10D
TNFRSF12A	AGTR1	IL11RA	LPHN1	AMFR	GPR161	TRAF6	
ANTXR1	MC1R	MELK	TRAF4	PTPRK	IFNGR1	IFT57	

Table III: Signaling pathways expressed across all microarray platforms and serum types

Notch Signaling
TGF-β Signaling
Wnt/catenin Signaling
Death Receptor
PPAR Signaling
IL-10 Signaling
p38 MAK signaling
NF- κ B Signaling
IL-6 Signaling
G-Protein Coupled Receptor Signaling
cAMP mediated Signaling

IV. DISCUSSION

Although there have been successful reports that support proliferation of MSCs using human serum [16, 18], which effectively eliminates risk of the xenogenic contamination, there has been no significant breakthrough that supports the proliferation of MSCs in serum-free medium [14]. The increasing therapeutic applications of MSCs accentuate the need for a serum-free supplement in order to support large-scale expansion and reproducible laboratory-to-laboratory conditions. The discovery of growth factors involved in MSC proliferation is key in developing the serum-free medium.

This study reported 94 HGRs and their corresponding 11 signaling pathways expressed during undifferentiated MSC proliferation. The 11 signaling pathways have been generally reported to be associated with cell proliferation and apoptosis. However, the exact role of each HGR and pathway in regulating cell proliferation and maintaining multipotency in MSC is unclear, although their consistent expressions across all four MSC cell cultures indicate key functions. The next step would be to investigate each HGR and signaling pathway individually in order to determine

more accurately the function of the pathways and consequently the role of each growth factor.

Besides growth factors, conventional FBS serum also contains a range of macromolecules, carrier proteins for lipid substances and trace elements, attachment and spreading factors, low molecular weight nutrients, and hormones [21]. These other components are critical for MSC proliferation and have to be defined in addition to the growth factors in order to develop a universally defined serum-free medium.

In this study, microarray data from three MSC cultures supplemented with FBS and one MSC culture supplemented with AS. It has been previously reported that serum supplement is a determinant of gene expression, with differential expression reflected from passage 4 [12]. When microarray analyses of MSC cell cultures were performed at passage 1, the difference in HGR expression was insignificant. Hence, it is possible that differential expression occurs only at later passages.

This study also reviewed the HGRs that were in agreement between the two platforms. This was done to ensure that the most expressed HGRs were represented. Another analysis involving all HGRs detected across each platform should be done. It would also be interesting to investigate the HGRs that were detected by the Affymetrix platform but not by the Illumina platform. While the expression levels of Notch ligands and receptors in undifferentiated MSCs are identified, it is helpful to compare these expression levels with differentiated cells further down the MSC lineage. We would be able to establish which ligands and receptors are differently regulated, and hence the function of the Notch Signaling pathway in MSCs.

In conclusion, the 94 HGRs and 11 signaling pathways were found to be expressed in undifferentiated MSCs. In the future, the exact roles of these signaling pathways and corresponding growth factors in maintenance of multipotency and proliferation in MSCs can be investigated and further developed into possible components in serum-free media for MSC expansion.

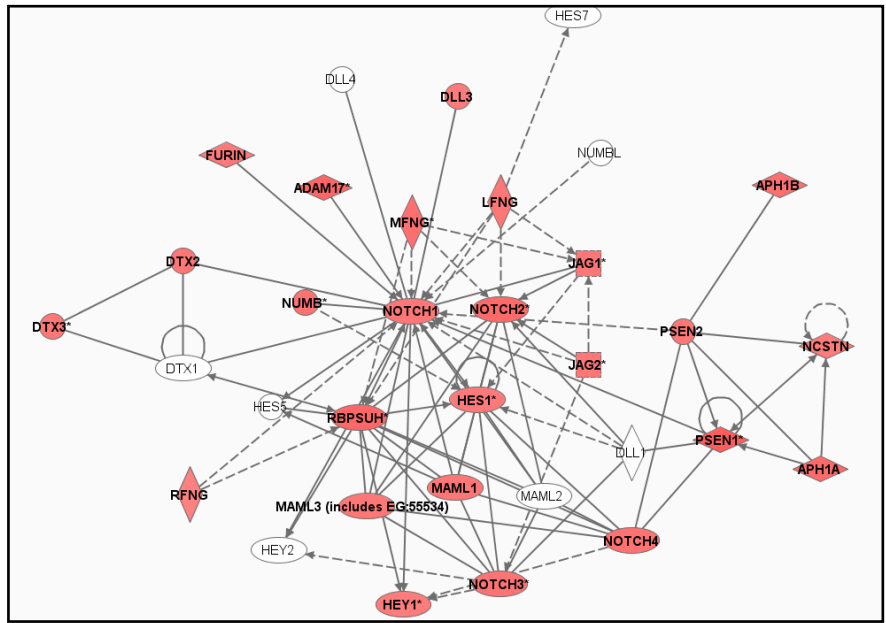


Figure 1: Overall expression of the Notch Signaling Pathway in the MSC sample cultured in AS and analyzed by Affymetrix

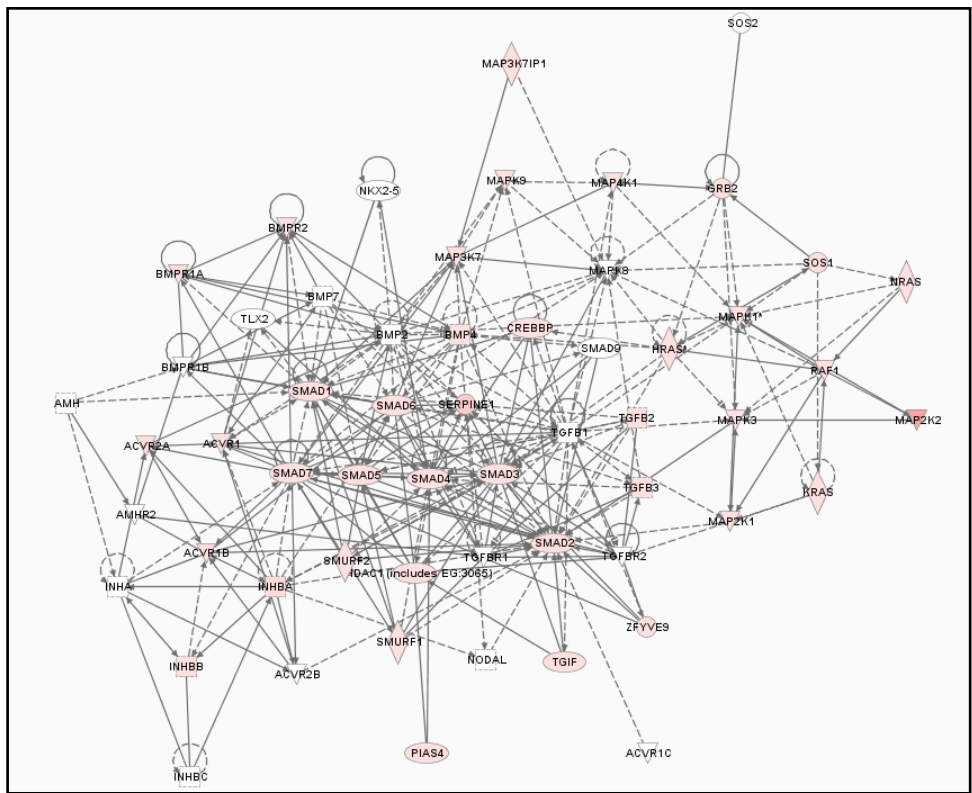


Figure 2: Overall expression of the TGF-beta Signaling Pathway in the MSC sample cultured in FBS and analyzed by Illumina

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