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MiRNA influences in mesenchymal stem cell commitment to neuroblast lineage development

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ABSTRACT

Mesenchymal Stem Cells (MSCs) are widely used in therapeutic applications. Their plasticity and predisposition to differentiate into a variety of cell types, including those of the neuronal lineage, makes them ideal to study whether a selection of miRNAs may direct the differentiation of MSCs into neuroblasts or neuroblastoma to mature neurons. Following a short-listing, miR-107, 124 and 381 were selected as the most promising candidates for this differentiation. MSCs differentiated into cells of the neural lineage (Conditioned Cells) upon addition of conditioned medium (rich in microvesicles containing miRNAs) obtained from cultured SH-SY5Y neuroblastoma cells. Characterisation of stemness (including SOX2, OCT4, Nanog and HCG) and neural markers (including Nestin, MASH1, TUBB3 and NeuN1) provided insight regarding the neuronal state of each cell type. This was followed by transfection of the three miRNA antagonists and mimics, and quantification of their respective target genes. MiRNA target gene expression following transfection of MSCs with miRNA inhibitors and mimics demonstrated that these three miRNAs were not sufficient to induce differentiation. In conditioned cells the marginal changes in the miRNA target expression levels reflected potential for the modulation of intermediate neural progenitors and immature neuron cell types. Transfection of various combinations of miRNA inhibitors and/or mimics revealed more promise. Undoubtedly, a mix of biomolecules is being released by the SH-SY5Y in culture that induce MSCs to differentiate. Screening for those biomolecules acting synergistically with specific miRNAs will allow further combinatorial testing to elucidate the role of miRNA modulation.

1. Introduction

Stem cell transplantation has been clinically proven to be both feasible and safe [1–3], and the novel approach of regenerative medicine by which tissues of organs are restored or gain normal function by the replacing, engineering or regenerating of the damaged cell [4] is also a novel approach to the treatment of neurological injury and disease.

Mesenchymal stem cells (MSCs) are multipotent stromal cells that are capable of self-renewal and can differentiate into a variety of cell types including osteoblasts, chondrocytes, myocytes and adipocytes [5]. Two properties of MSCs - their multipotentiality and ease of isolation - have shown the potential of playing major roles in tissue engineering and therapeutic applications. Different studies have shown how MSCs are able to differentiate into neural cell types either by the use of induction agents [6], or the use of conditioned media co-cultured with neural cells such as neurons, oligodendrocytes, and Schwann cells [7–9]. Conditioned medium includes a variety of biomolecules, including microRNAs (miRNAs) released by the cells into the culture medium during growth. These secreted biomolecules may play a role in processes such as cell growth, differentiation, invasion and angiogenesis by regulating cell-to-cell and cell-to-extracellular matrix interactions [10]. MSCs release microRNA-containing exosomes which are shuttled to neural cells, in turn regulating neurite outgrowth [11].

Over the years, the important role played by miRNAs in stem cell fate determination and differentiation has become more pervasive. More than 2600 miRNAs have been associated with the human genome [12,13] and most of these are expressed in the human brain [14]. With

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ongoing research, new miRNA/stem cell related functions are being discovered, which in turn may lead to developing new miRNA-based techniques applicable to therapies in regenerative medicine. It is well understood that cells can be reprogramed using miRNAs, however further elucidation is required on whether miRNAs alone can actually induce reprogramming or whether they are improving the efficiency of reprogramming factors [15]. A key aspect of miRNA modulation is identifying the roles of circulating miRNAs and how these may be made to good use as part of miRNA therapeutic applications. The potential use of miRNAs in diagnosis, prognosis and treatment of Neuroblastoma (NB) has been reviewed in Zammit et al. [16]. A miRNA therapeutic approach could be made possible by developing techniques which mimic stable exosomal miRNAs capable of efficiently delivering therapeutic miRNAs, which in turn will control the proliferation and differentiation of stem cells used as the basis for tissue regeneration [17]. The importance of the regulatory role that miRNAs play in differentiation and neural development was proven by eliminating Dicer or Drosha co-factor DiGeorge Syndrome Critical Region Gene 8 (DGCR8), which are key components of miRNA processes, by means of loss-of-function experiments [18–21]. Case in point, deviant miRNA expressions found in malignant NB cause a deregulation in epigenetic processes which in turn influence miRNA promoters [22].

This study investigated the role of three miRNAs - miR-107, miR-124 and miR-381 – in relation to how these can direct the differentiation of MSCs to neuroblasts to mature neurons. These miRNAs were shortlisted from a previous review (Zammit et al. 2018) and selected since the role of miR-107 in stem cells is not well defined, however this miRNA is well associated with the pathogenesis of human cancer [23]; Neurogenesis promotor, miR-124 is expressed in MSC-derived macrovesicles [24] and miR-381 induces neural stem cells differentiation to neurons [25], so once MSC have differentiated into neurons this miRNA should further differentiate these cells along the neuronal cell linage.

It is well known that miR-107 is specifically expressed in the brain [26] and acts as a negative regulator of dicer which implicates that this miRNA has a major role in the regulation of dicer-dependent physiological processes such as neurogenesis [27]. Its overexpression causes a loss of dicer, causing a reduction in brain morphogenesis and its downregulation stabilises dicer mRNA increasing the number of neuronal progenitors [27].

MiR-124 is a highly expressed tissue-specific miRNA of the nervous system [28–32]. In order to maintain the neural state, miR-124 downregulates the expression of non-neural mRNAs by repressing the expression of non-neural transcripts and thus directing the gene expression profile towards the neural state [33–35]. Upon cell lineage commitment, gene expression alteration is initiated by activating the expression of neuron-specific genes and repression of other genes that are not of the neuronal state.

MiR-381 targets Hes1, a gene highly expressed in the CNS, which plays a crucial role in the maintenance of neural stem cells (NSCs) during the development of the embryonic brain. Down-regulation of Hes1 will cause NSCs to differentiate into mature neurons [36] and over-expression of this gene inhibits their differentiation and proliferation [37]. Knockdown of Hes1 upregulates the neural differentiation factor Mash-1, increasing neuronal differentiation [38]. Overexpression of miR-381 inhibits Hes1 protein expression promoting NSC differentiation and proliferation to neurons and inhibits astrocyte differentiation [25].

It is widely agreed that miRNAs are responsible for neural induction, differentiation and fate specification and have thus begun to develop into a next generation therapeutic approach for many conditions such as NB. The identification of additional putative miRNAs responsible for the differentiation of MSCs towards neural development will further elucidate the mechanisms of action involved in both the physiological and pathological processes of the CNS.

2. Materials and methods

2.1. Cord collection and MSC culturing

Cord collection and MSC culturing was performed following the method previously described [39]. In brief, umbilical cord samples were obtained from healthy full-term neonates after informed consent was obtained from the mothers prior to delivery. A length of cord ranging between 5 and 10 cm was bled, placed in phosphate buffer saline (PBS) containing antibiotics (100units penicillin and 100 µg streptomycin/mL), and stored at a temperature of 2–8 °C. All samples were processed within 24 h of collection. After the cord had been cleaned with 70% ethanol, the epithelial and vascular tissues were removed, the Wharton's Jelly extracted and further digested using 0.5 mg/mL collagenase, after which tissue was incubated with complete medium (Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM-F12) supplemented with 20% cryo-poor plasma, 1% penicillin/streptomycin (10,000U/mL), and, N2 supplement) at 37 °C with 5% CO_2 in a humidified environment. Half the medium was replaced every three days. This allowed replenishing of nutrients and removal of debris whilst retaining some of the circulating growth factors released by the tissue itself. Once cells started to grow and colonies formed, the tissue fragments, together with the spent medium, were removed. Once confluence was achieved the cells were detached with 2 mM Ethylenediaminetetraacetic acid (EDTA) disodium salt dihydrate in PBS.

2.2. Preparation of conditioned medium and conditioning of MSCs

Conditioned medium was obtained by culturing SH-SY5Y cells in complete DMEM-F12 medium incubated at 37 °C with 5% CO_2 in a humidified environment. The medium was changed every 3 days and once confluent the spent medium was collected and centrifuged at 500 g for 15 min. Medium was stored at 2–8 °C for up to seven days.

MSCs were conditioned by the addition of equal volumes of complete medium and spent medium obtained from SH-SY5Y cultures. These were kept incubated at 37 °C with 5% CO_2 in a humidified environment and medium was changed every 3 days. Morphological changes could be seen within 24 h. Such cells are referred to hereafter as Conditioned Cells (CCs).

2.3. Characterisation for stemness properties

Cells were characterised for stemness markers by means of the Proteome Profiler™ Human Pluripotent Stem Cell Array (R&D Systems) that indicates the type of stem cells and the lineage into which they may differentiate. Once the cells were lysed and membrane prepared according to the manufacturer's instructions, the sample was added to the membrane and this was incubated overnight at 2–8°. The membrane was washed and an antibody cocktail added to it. This was again incubated, after which the membrane was again washed and Streptavidin-HRP was then added. This was left to incubate incubated for 30 min. Finally, anti-streptavidin antibody was added to the membrane and a visible reaction was noted within a few seconds.

2.4. Primer panel

2.4.1. End-point PCR

End-point PCR was performed to verify whether the cell types express the selected CD and neural markers (primer sequences in Tables 1 and 2 respectively) used in this study. PCR was performed using a set-up of initial heat activation of 2 min at 95 °C, denaturation at 94 °C, Annealing at 55 °C and extension at 72 °C, each for a duration of 10 s. On completion of 35 cycles, PCR products were run on a 1% agarose gel.

Table 1
List of primers for selected CD markers.

Primer	Forward	Reverse
CD14	CTGCAACTTCTCCGAACCTC	CCAGTAGCTGAGCAGGAACC
CD19	TACTATGGCACTGGCTGCTG	CACGTTCCCGTACTGGTTCT
CD34	TGAAGCCTAGCCTGTCAC	CGCACAGCTGGAGGTCTTAT
CD44	CCAATGCCTTTGATGGACCA	TGTGAGTGTCCATCTGATTC
CD45	GTGTTTCATCAGTACAGACG	GTTGTGGTTGAAATGACAGC
CD73	ATGGTGTGGAAGGACTGATC	CCTCACTTTCTGAGCGATG
CD90	TGCTCTTTGGCACTGTGG	AGAGGGAGAGCAGGAGCAG
CD105	GGGGTCAACACCACAGAG	CAGGACCCTCAGGATGTG
CD106	CCCTTGACCGGCTGGAGATT	CACAGGATTTTCGGAGCA
CD146	GAAAAAGTGTGGCTGGAAGT	GTTGTCGTTGGTTGTCTCTT
CD166	AGGAAATGGACCCAGTGAC	CCCCTTCTTTGATGGCA

Table 2
List of primers for selected Neural Markers.

Primer	Forward	Reverse
NES	TCCTGGAGGCTGAGAACTCC	CTGGCCAAGGTAGGGGTACG
SOX2	CAAGATGCACAACTCGGAGA	GGGCAGCGTGTACTTATCCT
ND1	TAAATTGAGACGCATGAAGG	GGTGGTGGGTTGGGATAAGC
TBR2	CACCTATCAGTACAGCCAGG	CTACGAACACATTGTAGTGG
MASH1	GAACTGATGCGCTGCAAACG	CATGCTCGTCCAGCAGCTGC
TUBB3	GGAGATCGTGCACATCCAG	TCGAGGCACGTACTTGTGAG
NeuN	TGTACACACCAGCACAGACC	CGAACATTTGCCGCAAGTCG
MAP2	ATACAGGGAGGATGAAGAGG	GGAGAAGGAGGCAGATTAGC
GAPDH	GAAGGTGAAGGTCGGAGTCA	GAAGATGGTGATGGGATTTC

Legend: SOX2 - SRY (sex determining region Y)-box 2, TUBB3 - β-III-Tubulin, NES – Nestin, ND1 – NeuroD1, TBR2 - T-box brain protein 2, MAP2 - Microtubule-associated protein 2, MASH-1 - mammalian achaete scute homolog-1, Neun1 - neuronal-specific nuclear protein 1.

2.5. End-point PCR for miRNAs

End-point PCR was also performed on selected miRNAs (see Table 3) using methodology described in section 2.4.1 described above.

2.6. Transfection

Cells were transferred to a 12-well plate and transfected once 70–90% confluent. Cells were transfected with mimics or antagomirs (and their respective scrambled controls) - as per Tables 5 and 6 respectively - using Mission® siRNA transfection reagent (Thermo Scientific). The plate was incubated for 24 h at 37 °C with 5% CO_2 in a humidified environment. After 24 h, total RNA was extracted, converted to cDNA and tested by RT-qPCR against a target gene (for each selected miRNA) and the neural marker panel (primer sequences in Tables 4 and 2 respectively).

2.7. RNA extraction and reverse transcription

Total RNA and miRNA were extracted from the cells using the SV Total RNA Isolation System (Promega Corporation) and mirVana miRNA Isolation Kit (Life Technologies) respectively. The cDNA was synthesised from the mRNA and miRNA using GoScript™ Reverse Transcriptase system (Promega Corporation) and TaqMan® MicroRNA Reverse Transcription Kit (Life Technologies).

Table 3
List of primers for selected miRNAs.

Primer	Forward	Reverse
miR-107	AGCAGCATTGT	TGATAGCCCTG
miR-124	TAAGGCACGC	GGCATTCACC
miR-381	ACAGAGAGCTT	TATACAAGGGC

Table 4
List of primers for selected miRNA Gene Targets.

Primer	Forward	Reverse
Dicer	CTTTCTTTGGACTGCCATGG	GTTGACCAAGAACACCGTCC
HES1	CCGGATAAACCAAAGACAGC	GGTGCTTCACTGTCATTTCC
Jagged-1	CAACACCTTCAACCTCAAGG	GGTCACGCGGATCTGATACT

Legend: HES1 – Hairy and enhancer of split 1.

Table 5
Mimics used for transfection.

Mimics	
Name	Sequence
has-miR-107 - MIMAT0000104	AGCAGCAGACAGGGCACA
has-miR-124-3p - MIMAT0000422	AAGGCACGCGGGAAGCC
has-miR-381-3p - MIMAT0000736	AACAAGGGCAAGCCCG

Table 6
Antagomirs used for transfection.

Anatgomirs	
Name	Sequence
simiR-107	GAAGCCCGACAAGCGC
simiR-124	ACAGAGAGCGCCCGAA
simiR-381	GGCACACCGCGGCCA

2.8. Quantitative PCR (qPCR)

The qPCR assay was performed using QuantiTect SYBR Green Rotor-Gene Q (Qiagen) and were normalised against GAPDH. During qPCR the cDNA was denatured for 15 s at 94 °C, annealed for 30 s at 50 °C and extended for 30 s at 72 °C. All primers were designed specifically to the human genome.

3. Results

3.1. Stem cell culturing

MSCs started to grow after 2 weeks and reached confluence within the next 15–20 days. Upon addition of the conditioned medium, MSCs differentiated within 24 h. Fig. 1 shows the transition from MSCs to CCs.

3.2. Characterisation for stemness properties

The Proteome Profiler™ Human Pluripotent Stem Cell was pre-spotted with 15 different antibodies printed in duplicate to detect stem cell markers. MSCs, CCs and SH-SY5y were tested, the results obtained being as shown in Fig. 2. MSCs were positive for Nanog, GATA-4, OTX2, VEGF R2/KDR/Flk-1, SOX2, HNF-3b/FoxA2, TP63/TP73L, HCG, E-Cad, PDX-1/IPF1, GSC, indicating their unique potential of differentiating into multiple cell lineages. CCs were positive for OTX2, HCG, and GSC, which indicate that these cells had started to lose their stemness properties. This is also shown by the greatly reduced intensity in the appearance of the spots. SH-SY5Y were negative for all markers probably due to the protein expression of any marker spotted on chip being absent or lower than the detection limit for this assay.

3.3. CD marker analysis

MSCs, CCs and SH-SY5Y were tested against known markers generally used for the characterisation of MSCs. These markers consisted of

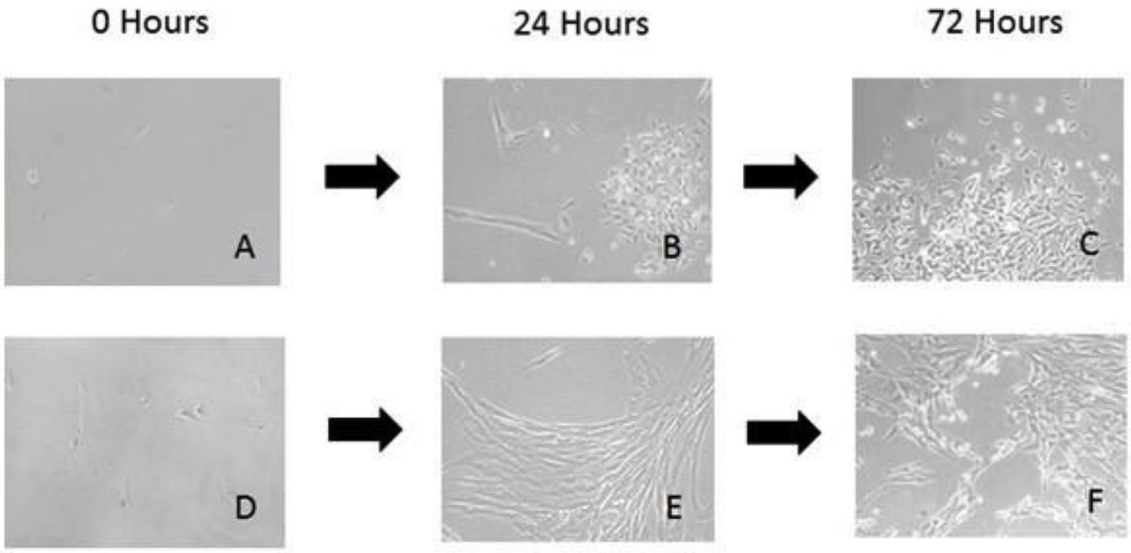

Fig. 1. Transition from Mesenchymal Stem Cells (MSCs) to conditioned cells. Conditioned medium was added to the MSCs and re-incubated (A). Within 24 h of adding conditioned medium, nearly all MSCs had differentiated into the neural cell lineage (B). Complete differentiation was obtained within the next 2–3 days (C). There was no change in flasks where fresh medium was added as control (D–F), which shows that cell differentiation was caused specifically by components secreted into the conditioned medium.

CD14, CD19, CD34, CD44, CD45, CD73, CD105, CD106, CD146 and CD166 and results obtained can be observed in Table 7 together with the expected results for MSCs and SH-SY5Y. MSCs followed a typical characterisation pattern except for CD14 and CD19, which are usually expected to be negative. SH-SY5Y cell characterisation was compared to the surface marker profile provided by Ref. [40]. While SHY-SY5Y cells are expected to be negative for CD14, CD34 and CD106, these three markers were positive in this study. Since CCs have been grown using medium in which SH-SY5Y have been cultured, it was expected that these two cell types would have a similar CD marker expression. Except for CD106, all other CD marker expressions of SH-SY5Y and CCs correspond and have a very similar level of intensity. Of interest is the switch from positive to negative in CD73, which is positive in MSCs but negative in both CCs and SH-SY5Y, and CD34 that is negative in MSCs and positive in CCs and SH-SY5Y. This switch in markers further confirms the transition from stem cells to cells of the neural lineage.

CD markers have been widely used to characterise MSCs [41]. Since these cells may be cultured from different sources and species, a wider range of markers are now being used to enable sorting of MSCs based on

Table 7
CD Markers detected.

CD Marker	MSCs	MSC Expected Results	Conditioned Cells	SH-SY5Y	SH-SY5Y Expected Results
CD14	+	–	+	+	–
CD19	+	–	+	+	–
CD34	–	–	+	+	+
CD44	+	+	+	+	+
CD45	–	–	–	–	–
CD73	+	+	–	–	–
CD90	+	+	+	+	+
CD105	+	+	+	+	+
CD106	+	+	–	+	+
CD146	+	+	+	+	+
CD166	+	+	+	+	+

Legend: CD – cluster of differentiation, MSCs – Mesenchymal Stem Cells, (+) – detected, (−) – not detected.

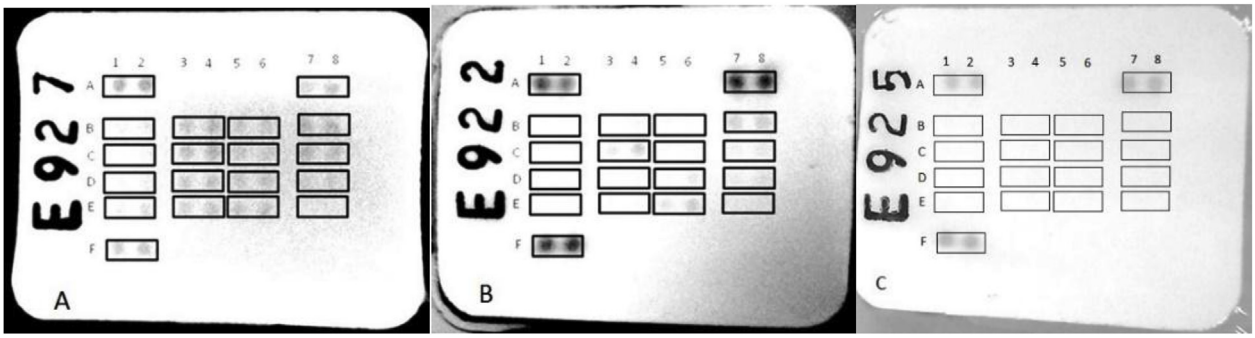

Fig. 2. Proteome Profiler Chip Results. MSCs (A) Conditioned Cells (B) and SH-SY5y (C) tested using the Proteome Profiler Human Pluripotent Stem Cell Array Kit. MSCs resulted positive for the following markers: Nanog, GATA-4, OTX2, VEGF R2/KDR/Flk-1, SOX2, HNF-3b, TP63/TP73L, HCG, E-Cad, PDX-1/IPF1, GSC. Conditioned Cells were positive for OTX2, HCG, and GSC. SH-SY5Y were negative for all markers. Negative control (E7,8) and reference spots A1,2, A7,8, and F1,2 validates the test.
Legend: MSCs – Mesenchymal Stem Cells, GATA-4 – GATA binding protein 4, OTX2 – orthodenticle homeobox 2, VEGF R2/KDR/Flk-1 – vascular endothelial growth factor receptor 2, SOX2 - SRY (sex determining region Y)-box 2, HNF-3b/FoxA2 – Hepatocyte nuclear factor 3-bet/Forkhead Box A2, TP63/TP73L – Tumour protein 63, HCG - human chorionic gonadotropin, E-Cad - E-Cadherin, PDX-1/IPF1 - Pancreatic and Duodenal Homeobox/Insulin Promoter Factor 1 and GSC – Goosescoid.

the expression of various CD markers. These CD characteristics are normally associated to BM-derived MSCs. However, identification of MSCs should not be limited to these since other sources possess less defined characteristics [42]. In fact, the MSCs cultured in this study showed positivity for CD14. A possible explanation for the positivity of CD14 is that the cells may express and epitope which causes a cross-reactivity when testing for CD14 [43].

Identifying MSCs by CD markers is not very reliable since cell surface markers have been inconsistently reported as being both positive or negative at one point or another [44]. CD markers are not a sole exclusive of MSC profiling but are also expressed by non-stem cells, with markers such as CD44 being also widely expressed on cancer cells [45,46]. Apart from cell surface markers, when identifying and categorising MSCs, the source of the tissue and method of isolation and expansion should be considered in conjunction with other characterising methods such as stemness properties, lineage and transcription factors [47].

The knowledge that MSCs should lack the CD34 expression is based on studies conducted on MSCs which were either cultured and grown as plastic adherent cells after several passages, after which they were later compared to haematopoietic stem cells which express the CD34 marker, or in studies which encouraged MSC culture and bone marrow was immuno-depleted with anti-CD34 [48]. In essence, studies using adipose-derived MSCs indicate that during the early stages of the isolation, MSCs are able to express CD34 [49]. A well-established fact is that MSCs dwell near or with blood vessels and their correlation to CD34 encourages the possibility that these cells are vascular stem cells [48]. The switch from a negative to positive and vice-versa expression may also be related to lineage commitment as cells start to differentiate into haematopoietic cells or endothelial cells [50,51].

3.4. Expression profiles of neural markers

As shown in Table 8, all three cell types expressed the selected markers at different levels of intensity. MSCs were highly reactive to SOX2, NeuroD1 and TBR2 while weak for TUBB3, MASH1, MAO2 and NeuN. CCs expression favoured Map2 and NeuN and were less reactive for Nestin, SOX2 and NeuroD1. Nestin, TUBB3 and MASH1 were best expressed by SH-SY5Y, while TBR2 was the least reactive.

The markers for the early neural epithelial characterisation used in this study were Nestin and SOX2. Nestin can be expressed by undifferentiated MSCs and is maintained even when these differentiate into other cell lineages [52]. The expression of Nestin in the MSCs cultured in this study was very similar to that found in the other two cell types. This might mean that MSCs have a predisposition to the neural stem cell lineage, whereas the CCs are still showing signs of early neural differentiation. Undifferentiated SH-SY5Y cells are known to express immature neuronal markers [53]. Since SOX2 acts both as a stemness marker and as an early neural marker, its presence may indicate the potential differentiation of the MSC cell lineage [54]. The expression of SOX2 in the three cell types is very similar. This sustained expression of SOX2 observed in CCs might indicate that these cells fall within the neuroepithelial lineage. Neuroblastoma cell types are known to have the capacity to express stemness markers such as Nestin, SOX2 and OCT4 [55], hence the detection in SH-SY5Y does not reflect an association with neuroepithelial cells. Both CCs and SH-SY5Y express SOX2 at a lower level than MSCs, although the amount of SOX2 expressed by SH-SY5Y is only slightly below that of MSCs.

TBR2 and MASH were used for the characterisation of Intermediate progenitors. TBR2 was the least reactive when compared to the other neural markers. As a result, the expression data for the TBR2 marker was considered as only being indicative of a trend and not fully reliable. MASH1 is expressed by poorly differentiated neuroendocrine carcinoma cells [56]. Neural marker MASH1 was expressed at similar levels by the three cell types. Having MSCs showing expression for this marker is comprehensible since downregulation of MASH1 activates the Notch signalling pathway that is responsible for retaining the properties of MSCs [57]. The expression of MASH1 in the CCs and SH-SY5Y might signify that these cells have the potential to differentiate further. SH-SY5Y are known to express MAP2 [58], which further indicate that these cells are fully differentiated.

Immature Neuron markers were characterised by TUBB3 and NeuroD1. TUBB3 was expressed by MSCs. This was much less than that expressed by both CCs and SH-SY5Y. However as described by Foudah et al. a high percentage of MSCs can spontaneously express this marker [59]. TUBB3 was expressed at similar levels by both the CCs and the SH-SY5Y suggesting a tendency towards immature neurons. NeuroD1 is expressed in well-differentiated cells of the neuroendocrine carcinoma lineage [60] and is an important regulator of NB [56]. All three cell types showed a similar expression for ND1.

Finally, MAP2 and NeuN markers were used to characterised cells found in the mature neuronal stage. The level of expression of MAP2 in CCs and SH-SY5Y is much higher than that expressed by MSCs. CCs expressed a very similar level of MAP2 as SH-SY5Y, meaning that these cells are also in the final stages of differentiation. Although MAP2 is normally a marker for mature neurons, it can also be expressed in MSCs [61]. In fact, MAP2 was found to be expressed by MSCs in this study, although the level of intensity detected is much lower than that observed in CCs and SH-SY5Y. MSCs spontaneously express the neural marker NeuN [59], whilst SH-SY5Y express this marker only once fully differentiated [58]. MSCs expressed this neural marker at lower levels than the other two cell types. Concomitantly NeuN was much more expressed in both CCs and SH-SY5Y, suggesting that both cell types have differentiated far enough to be considered mature neurons.

3.5. Presence of selected miRNAs

MiRNAs were relatively below the detection level of the qPCR testing. End-point PCR showed the presence of miRNAs as shown in Table 9.

MiR-107, 124 and 381 were all detected in CCs and SH-SY5Y but not in MSCs. Not being detected does not mean that these miRNAs are not expressed by these cell types and the conditioned medium. The amount of miRNA expressed by these cell types may be too low to be detected by such techniques. It is understood that if the cells *per se* have such a low abundance of miRNAs, the conditioned medium which should contain any secreted miRNAs would have an even lower abundance, making it much more difficult to detect.

Table 8
Neural markers.

Neural Marker	MSCs	Conditioned Cells	SH-SY5Y
Nestin	2+	1+	3+
SOX2	3+	1+	2+
NeuroD1	3+	1+	2+
TUBB3	1+	2+	3+
TBR2	3+	2+	1+
MASH1	1+	2+	3+
MAP2	1+	3+	2+
NeuN	1+	3+	2+

Legend: MSCs – Mesenchymal Stem Cells, (+) – detected, (−) – not detected.

Table 9
Presence of miRNAs.

MiRNA	MSCs	Conditioned Cells	SH-SY5Y
miR-107	–	+	+
miR-124	–	+	+
miR-381	–	+	+

Legend: MSCs – Mesenchymal Stem Cells, (+) – detected, (−) – not detected.

Table 10
Transfection of MSCs.

Mesenchymal Stem Cells				
Target Gene	Transfection	MiR-107	MiR-124	MiR-381
Dicer	Antagonist	–	↔	↑
Hes1		–	↓	↔
Jagged-1		–	–	–
PTBP1		↓	↔	↑

Legend: MSCs – Mesenchymal Stem Cells, ↓ - down regulation, ↔ - no change, ↑ - up regulation, (−) – Negative/not detected.

3.6. miRNA target genes after transfection of individual antagonists and mimics

Once transfected MSCs (Table 10), Dicer was over-expressed when cells were transfected with miR-381 antagonist and was absent in the presence of miR-107 antagonist. Transfection of miR-124 antagonist gave a similar result to that of the negative control meaning that Dicer was not affected. After transfection of miR-107 antagonist, the Hes1 gene signal decreased significantly and resulted below the detection limit. In the presence of miR-124 antagonist, expression of Hes1 was decreased whilst no change was noted between non-transfected cells and transfection of miR-381 antagonist. Jagged-1 was not detected in MSCs neither before nor after transfection of the three antagonists. Transfecting cells with miR-107 antagonists produced a decrease in PTBP1 expression, encouraging neural differentiation signalling. Although PTBP1 is a direct target of miR-124, no change in expression was seen after transfecting the antagonist, while transfection of miR-381 antagonist caused an increase in PTBP1 pointing towards the potential of MSCs of becoming early precursor neural cells.

On transfecting CCs (Table 11), Dicer expression was only slightly less expressed when transfected with miR-107 antagonist and no change was seen after transfection with miR- 124 antagonist was only slightly less expressed. An increase in expression was seen when transfected with miR-381 antagonist. Prior to transfection Hes1 was not detected in CCs. Once the CCs were transfected with the antagonists, both Hes1 and Jagged-1 expression was upregulated. Upregulation of PTBP1 was only seen with antagonist transfection of miR-124, and expression was downregulated with transfection of miR-107 and miR-381. Transfecting mimics in CCs caused a downregulation of Dicer and upregulated the expressions of Hes1, Jagged-1 and PTBP1.

Table 12 summarises the transfection of SH-SY5Y cells with antagonists and mimics. There was no difference in expression of Dicer in SH-SY5Y after transfection with miR-107 antagonist, while an increase was seen after transfection with miR-124 and 381 antagonists. Hes1 expression was downregulated by miR-107, increased with miR-124 antagonists and no apparent change was observed after transfecting miR-381 antagonist. Transfection of miR-107 and miR-124 antagonist upregulated Jagged-1 expression, which was in turn downregulated by miR-381 antagonist. PTPB1 was only affected by miR-107, which caused downregulation of this expression. Mimic transfection did not affect the expression of PTBP1. MiR-107 mimic downregulated expression of Dicer and upregulated Hes1 and Jagged-1. Gene expressions were upregulated by transfecting mIR-124. Transfection of miR-381 mimic upregulated Hes1 and Jagged-1 but did not have an effect on Dicer.

Table 11
Transfection of conditioned cells.

Conditioned Cells				
Target Gene	Transfection	MiR-107	MiR-124	MiR-381
Dicer	Antagonist	↓	↔	↑
Hes1		↑	↑	↑
Jagged-1		↑	↑	↑
PTBP1		↓	↑	↓
Dicer	Mimics	↓	↓	↓
Hes1		↑	↑	↑
Jagged-1		↑	↑	↑
PTBP1		↑	↑	↑

Legend: MSCs – Mesenchymal Stem Cells, ↓ - down regulation, ↔ - no change, ↑ - up regulation, (−) – not detected.

Table 12
Transfection of SH-SY5Y.

SH-SY5Y				
Target Gene	Transfection	MiR-107	MiR-124	MiR-381
Dicer	Antagonist	↔	↑	↑
Hes1		↓	↓	↔
Jagged-1		↑	↑	↓
PTBP1		↓	↔	↔
Dicer	Mimics	↓	↑	↔
Hes1		↑	↑	↑
Jagged-1		↑	↑	↑
PTBP1		↔	↔	↔

Legend: MSCs – Mesenchymal Stem Cells, ↓ - down regulation, ↔ - no change, ↑ - up regulation, (−) – Negative/not detected.

3.7. Overall effect of transfection

Fig. 3 shows the effect of transfection of miRNA antagonists on the different target genes during the differentiation of MSCs to neuronblasta. MiRNAs have multiple targets and so the selected targets of this study have not always shown the desired effect post-transfection of the antagonists. CCs showed a much more favourable outcome post-transfection particularly in view that the neuroblast cells used are cancerous and already committed to a set lineage, while MSCs have a much higher predisposition of differentiating into various cell types, other than those of the neuronal lineage.

Table 13 summarises the changes in both CCs and SH-SY5Y after transfection of antagonists when compared to MSCs. A decrease in miR-107 expression promotes neurogenesis and targets Dicer. A slight downregulation of Dicer can be observed in CCs upon transfection with the miR-107 antagonist, showing a possible increase in neurogenesis that, in turn, is pushing these cells towards becoming mature neurons. Jagged-1 a direct target of miR-124, whose presence promotes the proliferation of neural precursors. The increased expression of Jagged-1 found in CCs may signify that the neuronal state of these cells is that of the precursor stage. Another target of miR-124 is PTBP1 which when decreased neural differentiation is enhanced. The absence of change in CCs, confirms the Jagged-1 result of these cells being in the precursor stage. Presence of miR-381 targets Hes1 and promotes NSC proliferation and differentiation, so inhibiting this miRNA downregulates neurogenesis. The decreased expression of Hes1 in CCs supports the hypothesis that CCs may be in the neuronal precursor/intermediate stage. SH-SY5Y cells follow a similar trend but because these are a cancer cell line, it is difficult to observe a clear shift as numerous pathways are being dysregulated. All transfection caused some sort of shift however the most prominent shifts are always for the correct target.

Failed detection due to low RNA concentrations was ruled out by testing the samples against GAPDH. According to the cell type tested, each inhibitor produced similar results. Transfecting miR-107, miR-124 and miR-381 antagonists individually did not yield conclusive results. The possibility of MSCs producing a transcript variant of Jagged-1 or this being below detection level is more plausible than MSCs not expressing Jagged-1, as it is highly improbable for these cells not to express an important ligand of the Notch signalling pathway. CCs seem to be the ones that are most susceptible to gene expression changes via miRNA manipulation. The reason why the target genes in SH-SY5Y

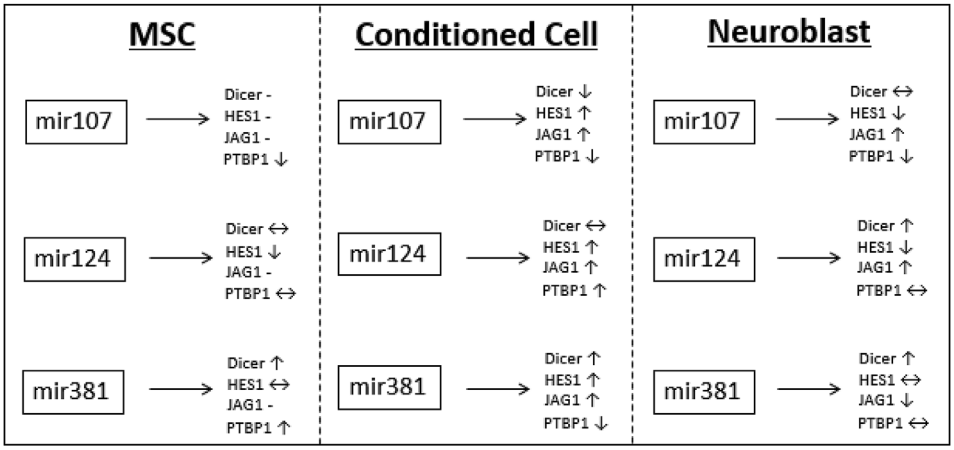

Fig. 3. Effect of transfection of miRNA antagonists on the different target genes during the differentiation of MSCs to neuronblasts.

Table 13
Comparison between the target gene expression of Conditioned Cells and SH-SY5Y with that of MSCs.

Target Gene	Conditioned Cells	SH-SY5Y
si 107 Dicer	↓	↔
si 124 JAG	↑	↑
Si 124 PTBP1	↔	↑
Si 381 Hes1	↑	↔

Table showing the effect on the target gene expression after transfecting cells with miRNA antagonists, when these where compared with their respective negative control.
Legend: MSCs – Mesenchymal Stem Cells, ↓ - down regulation, ↔ - no change, ↑ - up regulation.

were not being affected by silencing miRNAs might be due to these cells being more resistant to transcriptional regulation. MiRNAs have multiple targets and the result of a transfection is the net change of all the downregulated genes, so there might be a number of proteins acting against each other, therefore the desired knockdown or upregulation is not observed, or the change is not as large as expected.

3.8. miRNA target genes post-transfection with a combination of mimics and antagonists

Fig. 4 shows the differentiation from MSCs to CCs (as a result of the addition of spent medium to the cells in culture) and the transition of CCs to neuroblasts and further to mature neurons by means of transfecting a combination of both miRNA antagonists and mimics. The change in gene target expressions suggests that combining miR-107 and miR-381 mimics with miR-124 antagonist encourages the differentiation of CCs into neuroblasts and finally into mature neurons. Transfection of combined miR-107 and miR-381 mimics seems to block neural differentiation in the neuroblastic stage at the same time causing these cells to revert back to the neural stage of CCs.

As seen in Table 14, there is a decrease in the Dicer gene expressed by CCs after transfection of combined mimics and antagonists causing a decrease in Neurogenesis directing cells to differentiate beyond their precursor stage. The increase in expression of Dicer in SH-SY5Y after transfection may indicate that transfection was not affective and that cells are in a transit stage, although on the other hand Dicer is a generic gene and is easily overexpressed. Hes1 expression is reduced both in CCs and SH-SY5Y after transfecting combined mimics and antagonists. The reduced expression of Hes1 signifies a decrease in neural stem cell proliferation and differentiation that in turn indicates that both these cell types are beyond the neural precursor stage. Although not very effective, the transfection of the combination of mimics and antagonist has produced an increase in expression of Jagged-1 in CCs. However, the same transfection in SH-SY5Y shows a decrease of the same gene expression. While the increase of Jagged-1 in CCs favours these cells as being still in their precursor stage because of an increase in the proliferation of neuronal precursors, the decrease of this target gene in SH-SY5Y supports the fact that these cells are further down the neuronal spectrum. A decrease in PTBP1 was seen in SH-SY5Y after transfection of combined mimics and antagonists thus promoting neural differentiation which in turn shows that these cells are moving further down the neuronal lineage. The increase in PTBP1 expression in the CCs supports the idea that these cells are in their precursor stage.

Nestin was selected because it is an early neuronal lineage marker. It is expected that with a decrease in miR-107 neurogenesis is promoted and that an increase in miR-124 and miR-381 will promote respectively neural progenitor proliferatio, NSC proliferation and differentiation. The resultant expression of Nestin post-transfection is shown in Tables 15 and 16.

Legend: MSCs – Mesenchymal Stem Cells, ↓ - down regulation, ↑ - up regulation, 1 - miR-107 and miR-381 mimics with miR-124 antagonist, 2 - miR-381 mimic with miR-124 antagonist, 3 - miR-107 mimic with miR-124 antagonist, 4 - miR-107 and miR-381 mimics.

The decrease in Nestin expression in the CCs after transfection of miR-107 and miR-381 antagonist, showed that neurogenesis was inhibited and that these cells were in the early neuronal precursor stage. Transfection of miR-124 antagonist increased Nestin expression, implying that neural progenitor proliferation was stopped, blocking the CCs in their early precursor stage. When transfected with the miRNA mimics and a combination of mimics and antagonist, Nestin expression was enhanced. This points out that CCs tend towards being between the early neural precursor and intermediate neuronal stage and that the net effect of transfection depends on the particular neuronal stage in which the CCs are at that moment. In MSCs, a decrease in Nestin was observed following transfection with miR-107 and miR-124 antagonist, while the miR-381 antagonist transfection showed a significant increase in Nestin expression. Since these are stem cells they proliferate well in the presence of miR-381. Transfecting SH-SY5Y with mimics or antagonist did not affect Nestin expression. However, when these cells were transfected with a combination of mimics and antagonists, Nestin decreased indicating that these cells had matured further and moved beyond their precursor stage.

4. Discussion

MSCs originate from the mesodermal germ layer [62] and were initially isolated from Bone Marrow (BM) [48], as fibroblastic colony-forming units, [63] and later cultivated from other different types of tissues [48]. The International Society for Cellular Therapy attribute

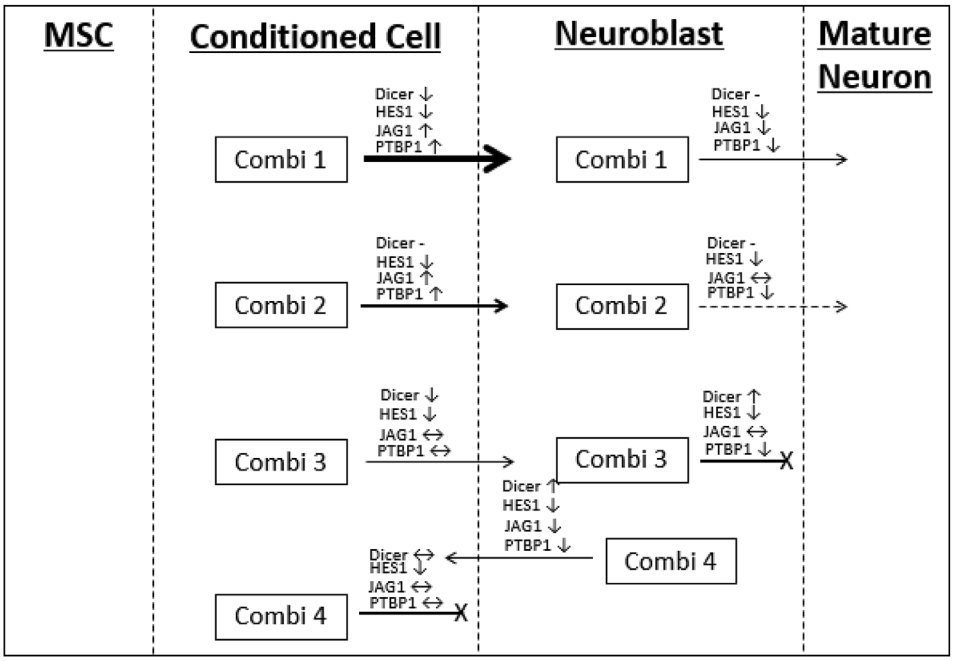

Fig. 4. Differentiation of MSCs into neuroblasts or neuroblastoma to mature neurons.
Legend: MSCs – Mesenchymal Stem Cells, ↓ - down regulation, ↔ - no change, ↑ - up regulation, (−) – negative/not detected, 1 - miR-107 and miR-381 mimics with miR-124 antagonist, 2 - miR-381 mimic with miR-124 antagonist, 3 - miR-107 mimic with miR-124 antagonist, 4 - miR-107 and miR-381 mimics.

Table 14
Target gene expression post combination of mimic and antagonist transfections.

Transfected cells	Target	1	2	3	4
Conditioned Cells	Dicer	↓	–	↓	↔
	Hes1	↓	↓	↓	↓
	Jagged-1	↑	↑	↔	↔
	PTBP1	↑	↑	↔	↔
SH-SY5Y	Dicer	–	–	↑	↑
	Hes1	↓	↓	↓	↓
	Jagged-1	↓	↔	↔	↓
	PTBP1	↓	↓	↓	↓

Legend: MSCs – Mesenchymal Stem Cells, ↓ - down regulation, ↔ - no change, ↑ - up regulation, (−) – negative/not detected, 1 - miR-107 and miR-381 mimics with miR-124 antagonist, 2 - miR-381 mimic with miR-124 antagonist, 3 - miR-107 mimic with miR-124 antagonist, 4 - miR-107 and miR-381 mimics.

Table 15
Nestin expression post antagonist and mimic transfections.

Nestin Expression				
Cell Type	Transfection	MiR-107	MiR-124	MiR-381
MSCs	Antagonist	↓	↓	↑
Conditioned Cells		↓	↑	↓
SH-SY5Y		↓	↔	↔
Conditioned Cells	Mimics	↑	↑	↑
SH-SY5Y		↔	↔	↔

Legend: MSCs – Mesenchymal Stem Cells, ↓ - down regulation, ↔ - no change, ↑ - up regulation.

Table 16
Nestin expression post combination of antagonist and mimic transfections.

Nestin expression post combined mimic and antagonist transfection				
Transfected cells	1	2	3	4
Conditioned Cells	↑	↑	↑	↑
SH-SY5Y	↓	↓	↓	↓

three minimum standards required by a cell to be identified as multipotent mesenchymal stromal cell [64] these being: plastic adherent; CD105, CD73 and CD90 positive, and negative for CD45, CD34, CD14 or CD11b, CD79alpha or CD19 and HLA-DR surface molecules and; their ability to differentiate *in vitro* into osteoblasts, adipocytes and chondroblasts.

Other studies have shown that MSCs derived from bone marrow [65,66] and adipose tissue [67,68] can be made to differentiate into neural cells. In this study, umbilical cord-derived MSCs are differentiating into cells of the neuronal lineage by the addition of spent medium obtained from SH-SY5Y cells in culture. Apart from morphological changes, when comparing stemness markers, it is noted that CCs retain neural stemness markers OTX2 and GSC but lose all other markers for which the MSCs had tested positive. This change was also confirmed by the change from negative to positive of CD34 and positive to negative of CD73, which in turn correspond to those expressed by SH-SY5Y. Clearly something is causing this differentiation, and this aimed to elucidate whether the selected miRNAs are in part responsible for bringing about this change.

To understand the potential neuronal stage of the MSCs and CCs, these cells where tested for a series of neuronal markers associated with the early neural epithelial, intermediate progenitors, immature and mature neurons stages. In the absence of specific differentiating agents, MSCs can express neural markers which in turn confirms their predisposition to differentiate into cells of non-mesengenic lineages such as neurons [61]. Neural lineage markers are expressed by cells that are formed during neurogenesis and help distinguish between these cells having a neural phenotype and other brain cell types [69]. The MSC and CC lineage express neural markers to different extents. Of interest is the fact that CCs express neural markers that are very similar to SH-SY5Y, which confirms that differentiation of these cells was induced by the conditioned medium. CCs are at a stage where phenotypically they are shifting towards mature neurons and neuroblasts (like the SH-SY5Y cell line), whilst still retaining some of the MSC features. This mixture of neural cell characteristics shows how MSCs, once triggered by the addition of conditioned medium, start to differentiate into neural epithelial cells, going on to become immature neurons, then intermediate progenitors, and finally reaching the stage of mature neurons. When still at an early stage of differentiation, SH-SY5Y grow in clusters [60] with a tendency of continuous proliferation expressing immature neural

markers [70]. Once these cells start to mature and differentiate, the proliferation rate decreases (Encinas et al., 2000; Påhlman et al., 1984) and they start to express mature neuronal markers such as TUBB3, MAP2, NeuN, synaptic associated protein-97 and NSE [53,72], whilst lacking the expression of glial markers such as glial fibrillary acidic protein and Nestin [71]. Following transfection, analysis of a wide variety of neuronal markers is required to confirm the degree of shift that would have occurred in the neural stage. However, testing of neural markers prior to transfection did not yield any satisfactory results as there is no clear cut between one stage and other as all cell types have shown reactivity of various degrees to all markers. Thus, to determine whether cells are either early neuroblast precursors or have indeed differentiated onwards, only Nestin was tested after transfection.

After transfection, a marginal change in the targets was observed in CCs showing potential for the modulation of intermediate neural progenitors and immature neuron cell types. Transfecting CCs with miR-107, 124 and 381 mimics showed a decrease in Dicer and an upregulation of Jagged-1 and PTBP1, and Hes1 respectively. A not so clear distinction was seen with transfection of antagonists as Jagged-1, PTBP1, and Hes1 all showed to be upregulated once transfected. Transfecting CCs with a variety of mimics and antagonists results in promotion of neurogenesis and decreases stem cell differentiations, possibly reverting these cells to an earlier stage. On the other hand, MSCs were only responsive to transfection of miR-107 antagonist which resulted in a downregulation of Dicer. However, this was to be expected since MSC have the potential of differentiating into neural cells and a decrease in Dicer promotes the production of neural progenitors. The increase in Nestin expression of CCs once these were transfected with mimics shows the potential of pushing these further down the neuronal cell lineage.

To better elucidate the role that miRNAs play in the differentiation of MSCs which is brought about by the addition of spent medium is analysing the actual composition of the medium used. Identifying the difference in miRNA levels found in the medium used for cell culture and the conditioned medium may help explain how this change, if any, may cause the MSCs to differentiate into cells of the neural lineage. Finally, to confirm upregulation or knockdown of the gene targets, it is recommended to perform functional microarrays to determine knockdown or upregulation of these target genes.

5. Conclusion

MiRNAs are responsible for neural induction, neuronal differentiation and fate specification and thus have begun to develop into a next generation therapeutic approach for many conditions such as NB. Stem cells have a well-established role in therapeutic approaches and, by combining stem cells, putative miRNAs responsible for the neural development can further elucidate the mechanisms of action involved in both the physiological and pathological processes of the CNS. The aim of this study was to identify if the three selected miRNAs by themselves or in different combinations may direct differentiation of MSCs to become neuroblasts or further down the neuronal cell lineage. Transfection with the selected inhibitors showed that these miRNAs on their own are not sufficient to induce MSCs to differentiate. In most cases the observed change for the selected neuronal markers in CCs was sub-optimal and therefore not reliably indicative due to the variability obtained during the testing of neural markers. Thus, testing by the selected panel of neural markers with the exception of Nestin, was deemed to not reflect reliably the change observed. Other neural markers need to be tested, specifically for detecting the changes of interest in CCs.

Further studies will explore why MSCs are being differentiated along the neuronal lineage by the addition of the SH-SY5Y spent medium. There are undoubtedly other biomolecules are being released by the SH-SY5Y in culture that induce MSCs to differentiate. Once a number of these substances are identified, further testing will be required to elucidate how these biomolecules synergistically interact to bring about miRNA modulation.

Authors' declaration

The authors declare there is no conflict of interest.

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