Functional Impact of Heterogeneous Nuclear Ribonucleoprotein A2/B1 in Smooth Muscle Differentiation from Stem Cells and Embryonic Arteriogenesis

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Running Title: hnRNPA2/B1 promote Smooth Muscle Differentiation

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Capsule

Background
SMC differentiation is a complicated process involving many transcription factors.

Results
hnRNPA2/B1 regulates SMC differentiation gene expression transcriptionally, and promotes neural crest cell migration and differentiation towards SMCs.

Conclusions
hnRNPA2/B1 promotes smooth muscle differentiation and development in vitro and in vivo.

Significance
This is the first report to demonstrate the functional involvements of hnRNPA2/B1 in SMC differentiation from stem cells and embryonic arteriogenesis.
ABSTRACT
Heterogeneous nuclear ribonucleoproteins (hnRNPs) play various roles in transcriptional and posttranscriptional modulation of gene expression. However, it remains unclear if hnRNPs are associated with smooth muscle cell (SMC) differentiation from stem cells and embryonic arteriogenesis. In the present study, mouse embryonic stem (ES) cells were cultivated on collagen IV-coated plates and smooth muscle differentiation medium. We found that hnRNPA2/B1 gene and protein expression was significantly up-regulated following 3 to 7 days of cell differentiation. hnRNPA2/B1 knockdown resulted in down-regulation of specific smooth muscle markers and transcription factors, while enforced expression of hnRNPA2/B1 enhanced the expression of these genes. Moreover, we demonstrated using luciferase and chromatin immunoprecipitation assays that hnRNPA2/B1 could transcriptionally regulate SMC gene expression through direct binding to promoters of SMαA and SM22α genes. We further demonstrated that chromobox protein homolog gene 3, a previously indentified SMC differentiation regulatory nuclear protein, is required for hnRNPA2/B1-mediated SMC differentiation genes expression. Importantly, specifically designed hnRNPA2/B1 morpholino for in vivo knockdown could inhibit the migration and differentiation of neural crest cells into SMCs in chick embryos. This resulted in the maldevelopment of branchial arch arteries and increased embryo lethality at a later developmental stage. Our findings demonstrated that hnRNP A2/B1 plays a functional role in SMC differentiation from stem cells in vitro and embryonic branchial arch artery development. This indicates that hnRNPA2/B1 is a potential modulating target for deriving SMCs from stem cells and cardiovascular regenerative medicine.

Key words: heterogeneous nuclear ribonucleoprotein A2/B1, stem cells, smooth muscle cells, cell differentiation, chick embryo, arteriogenesis.

INTRODUCTIONS
Understanding signalling pathways and molecular mechanisms in regulating stem/progenitor cell differentiation into cardiomyocytes, endothelial and smooth muscle cells (SMCs) are crucial. This is especially so for cardiovascular regenerative medicine which eventually benefits patients in clinical applications. Unlike endothelial cells and cardiomyocytes, which are terminally differentiated, adult SMCs retain significant plasticity in order to carry out different functions such as proliferation, migration, and synthesis of extracellular matrix protein in a series of cardiovascular pathogenesis(1). Therefore, studies focused on signalling pathways and molecular mechanisms in SMC differentiation are crucial for improving our understanding of the pathological process of cardiovascular diseases and developing novel therapeutic strategies for these diseases. Although the regulatory networks and transcriptional regulators of SMC differentiation have been extensively
investigated in the recent years by our group and other researchers(2-13), whether certain heterogeneous nuclear ribonucleoproteins (hnRNPs) are involved in SMC differentiation genes regulation remains obscure.

hnRNPs belong to the RNA-binding protein family and play important roles in regulating gene expression at both transcriptional and posttranscriptional levels(14). Each hnRNPs includes at least one RNA-binding domain such as RNA recognition motif (RRM), hnRNP K homology domain (KH) or arginine/glycine-rich (RGG) box(14). The significance of these RNA-binding domains have been strongly linked to its importance in gene posttranscription, mature mRNA cytoplasm transportation and protein translation through its binding to pre-RNAs(15,16). However, accumulating evidence also suggests that RRM and RGG of hnRNPs are involved in the binding of single-stranded DNA(17), telomere biogenesis(18,19) and transcriptional regulation(20,21). For example, it has been reported that hnRNPA2/B1 can bind to the promoters of c-myc, APOE, vitamin D receptor, breast cancer 1 and gonadotropin-releasing-hormone 1 genes and regulate these genes expression(22-26). However, to date, there has been no report of the functional involvement of hnRNPA2/B1 in SMC differentiation and embryonic development. In the present study, we first identified an essential role of hnRNPA2/B1 in SMC differentiation from embryonic stem (ES) cells. We demonstrated that hnRNPA2/B1 regulated the expression of SMC differentiation genes by directly binding to and activating its promoters of specific SMC genes such as SMαA and SM22α. In addition, we further demonstrated for the first time that knockdown of hnRNPA2/B1 by using specifically designed hnRNPA2/B1 morpholino could disrupt chick neural crest cell migration and differentiation into branchial arch arterial SMCs. Our findings highlight the importance of hnRNP A2/B1 for SMC differentiation from both stem cells and neural crest cells.

Materials and Methods

**Cell culture and SMC differentiation.** Detailed protocols for mouse ES cell (ES-D3 cell line, CRL-1934; ATCC, Manassas, USA) culture and SMC differentiation were described in our previous studies(2-8). For SMC differentiation, undifferentiated ES cells were seeded on mouse collagen IV (5μg/ml)-coated flasks or plates in differentiation medium [DM, MEM alpha medium (Gibco) supplemented with 10% FBS, 0.05mM β-mercaptoethanol, 100 U/ml penicillin, and 100 μg/ml streptomycin] for 3 to 7 days prior to further treatments. The medium was refreshed every other day.

**hnRNPA2/B1 expression plasmids generation.** Mouse and Gallus full length hnRNPA2/B1 gene was amplified using RT-qPCR from day 3 differentiating ES cells and chick embryos with primer set as shown in Table S1 and cloned into Kpn I/Pst I sites of the pCMV5-HA and pSPT18 expression
vector, designated as pCMV5-HA-hnRNPA2/B1 and pSPT18-hnRNPA2/B1. All the vectors were verified by DNA sequencing.

**Nucleofection, siRNA experiments, indirect immunofluorescent staining for cells, real-time quantitative PCR (RT-qPCR) were performed as previously described**.(2-8)

**Chromatin immunoprecipitation (ChIP) assays.** As previously described(3,5,7,8), differentiating ES cells transfected with pCMV5 or pCMV5-hnRNPA2/B1 were treated with 1% (v/v) formaldehyde at room temperature for 10 min and then quenched with glycine at room temperature. After removing the medium, cells were harvested for sonication. The sheared samples were diluted into 1 ml immunoprecipitation buffer containing 25 mM Tris-HCl, pH 7.2, 0.1% NP-40, 150 mM NaCl and 1 mM EDTA. Immunoprecipitation was conducted with antibody raised against hnRNPA2/B1, together with single-strand salmon sperm DNA saturated with protein-G-Sepharose beads. Normal IgG was used as a control. The immunoprecipitates were eluted from the beads using 100 μl elution buffer (50 mM NaHCO3, 1% SDS). A total of 200 μl proteinase K solution was added to a total elution volume of 300 μl and incubated at 60°C overnight. Immunoprecipitated DNA was extracted, purified, and then used to amplify target DNA sequences by real-time quantitative PCR (RT-qPCR). Relative DNA level was defined as the ratio of immunoprecipitated promoter DNA level to its input level with that of the control sample (pCMV5) set as 1.0. Target DNAs were almost undetectable in the normal IgG control samples. The data was obtained from three independent experiments.

**Chick embryo cultivation and staging.** Fertilized chick eggs were obtained from Joyce and Hill (Farm) and incubated at 38.5°C in a humidified incubator. Hamburger and Hamilton (HH) staging was applied throughout the study(27,28).

**Whole-mount immunohistochemistry and in situ hybridization were carried out as previously described**(7).

**In vivo electroporation on chick embryos.** *In vivo* electroporation carried out on chick embryos was performed as previously described but with some modifications(7). Eggs were incubated in a humidified incubator at 38°C for around 37 hours. A window was opened at the top of the egg shell using a blade, and embryos at HH 10/10+/10+ were electroporated with hnRNPA2/B1 morpholino (1μM, 5’TCTCTCTGTCGCCGCGAGGCATCTT3’, Gene Tools, LLC) or control morpholino (1μM, 5’CCTCTTACCTCAGTTACAATTTATA3’). Electroporator was set at 7.5 volts, 4 pulses, length of 50ms and interval of 1000ms. Oligonucleotides were mixed with 60% sucrose before electroporation. After electroporation, the eggs were sealed well and placed back into the incubator, window side
up, and incubated until the required developmental stages.

**Statistical analysis.** Data were expressed as mean±SEM and analyzed using two-tailed student’s *t*-test for two-groups comparison or one-way ANOVA for comparing different groups. Chi-Square analysis was performed using SPSS 17.0 software to compare the percentage of embryonic maldevelopment in two groups. A value of *P* < 0.05 was considered statistically significant.

For detailed materials and methods, refer to online supporting information.

**Results**

**hnRNPA2/B1 is involved in ES cell differentiation to SMC**

In parallel with SMC specific gene inductions as reported previously(2-8), the expression levels of hnRNPA2/B1, one of the several up-regulated nuclear proteins found during early SMC differentiation as demonstrated by our nuclear proteomics analysis (data not shown), were transiently elevated when ES cells were cultivated on collagen IV-coated plates in the absence of leukaemia inhibitory factor to allow for SMC differentiation (**Figure 1A and 1B**). The levels of hnRNPA2/B1 expression peaked at day 3 and displayed a sustained signal until day 7, suggesting that hnRNPA2/B1 may play a role during ES cell differentiation to SMC (**Figure 1A and 1B**). As expected, immunofluorescent staining analyzed using confocal microscopy further confirmed the nuclear location of hnRNPA2/B1 proteins in differentiating cells (**Figure 1C**).

**Essential roles of hnRNPA2/B1 in SMC differentiation from ES cells**

In order to determine the role of hnRNPA2/B1 in SMC differentiation, the effects of endogenous hnRNPA2/B1 knockdown on the expression of SMC specific genes were examined. It showed that the knockdown of endogenous hnRNPA2/B1 gene expression significantly decreased mRNA expression of multiple SMC-specific differentiation genes (SMαA and SMMHC) and transcription factors (SRF and Myocardin) (**Figure 2A**). Similarly, Western blot analysis also revealed decreased protein levels of these genes in hnRNPA2/B1 knockdown cells (**Figure 2B and 2C**). To further investigate if hnRNPA2/B1 expression is sufficient for specific SMC gene expression, we generated hnRNPA2/B1 expression plasmids. It is noteworthy that only the A2 isoform of hnRNPA2/B1 was successfully generated in our study as demonstrated by DNA sequencing of the expression plasmids (data not shown). Overexpression carried out using different amounts of hnRNPA2/B1 plasmids was conducted in differentiating ES cells. RT-qPCR and Western blot analyses revealed that overexpressing hnRNPA2/B1 significantly increased SMC gene expressions at both mRNA (**Figure 2D**) and protein (**Figure 2E**) levels in a dose-dependent manner. Importantly, gene expression levels of SMC transcription factors were also dramatically increased in overexpressing cells (**Figure 2E**).
and 2F). Taken together, the above findings strongly suggest that hnRNPA2/B1 gene expression is essential for SMC differentiation from stem cells.

**hnRNPA2/B1 mediated SMC differentiation through transcription activation**

As demonstrated above, hnRNPA2/B1 influences the levels of SMC marker expression in differentiating ES cells. In order to understand how hnRNPA2/B1 regulates SMC gene expression, day 3 differentiating ES cells were transfected with pCMV5 or pCMV5-hnRNPA2/B1 and treated at day 5 with the RNA synthase inhibitor, actinomycin D (1µg/ml) for 6 hours. Total RNA was harvested and subjected to RT-qPCR analysis to examine SMαA and SM22α gene expression. Treatment with actinomycin D ablated the effect of hnRNPA2/B1 overexpression on SMαA and SM22α gene expression (Figure 3A), which suggested that hnRNPA2/B1 may activate SMαA and SM22α gene expression at a transcriptional level.

To further test our hypothesis that hnRNPA2/B1 activates the specific SMC gene transcription, luciferase gene reporter and ChIP assays were performed. The plasmids used in the luciferase assay, pGL3-SMαA-Luc and pGL3-SM22α-Luc, were previously designed and cloned in our laboratory(3). ES cells were cultured on collagen IV-coated 12 well plates and co-transfected at day 3 of differentiation. The cells were either transfected with hnRNPA2/B1 overexpressing vector (400ng/5×10⁴ cells) or the control vector, alongside with the luciferase reporter gene under the control of SMαA or SM22α promoters. pGL3-Renilla vector was included in all samples as an internal control to assess transfection efficiency. The Luciferase activity and Renilla signal were detected 48 hrs after transfection. Data shown in Figure 3B revealed that the overexpression of hnRNPA2/B1 in differentiating ES cells significantly increased SMαA or SM22α promoter activities. This indicates that hnRNPA2/B1 directly activates specific SMC gene promoters. Furthermore, ChIP assays were conducted using an hnRNPA2/B1 antibody in the differentiating ES cells to further verify if hnRNPA2/B1 activates specific SMC gene transcription through direct binding to their promoters. It revealed that hnRNPA2/B1 directly bound to the promoters of SMαA and SM22α (Figure 3C), and such binding was dramatically enhanced by hnRNPA2/B1 over-expression. These findings demonstrate for the first time that hnRNPA2/B1 regulates SMαA and SM22α gene expressions during SMC differentiation through direct binding to the promoter region of specific SMC genes.

**hnRNP A2/B1 regulates Cbx3 gene expression**

We have previously reported that nuclear protein Cbx3 plays a crucial role in SMC differentiation from stem cells and chick
embryonic arteriogenesis(7). Interestingly, by examining the genomic locations of hnRNPA2/B1 and Cbx3, we noticed that both mouse Cbx3 and hnRNPA2/B1 gene localize in chromosome 6 and are closely linked to each other(29). In particular, the 5’-upstream region of Cbx3 gene exhibits a high degree of similarity to a portion of hnRNPA2/B1 gene. Therefore, we wondered whether there is a relationship between these two genes. To test our hypothesis, knockdown experiments were conducted with Cbx3 and hnRNPA2/B1 siRNAs in differentiating ES cells. The levels of gene and protein expressions of both genes were then analyzed using RT-qPCR (Figure 4A) and Western blot analysis (Figure 4B). Data shown in Figure 4A and B revealed that both Cbx3 gene and protein levels were significantly downregulated by the knockdown of Cbx3 gene itself as well as by the knockdown of the hnRNPA2/B1 gene. Neither gene nor protein expression of hnRNPA2/B1 was downregulated by the knockdown of Cbx3 gene, suggesting that Cbx3 acts downstream of hnRNPA2/B1. Importantly, overexpression of hnRNPA2/B1 alone significantly upregulated Cbx3 gene expression at both mRNA (Figure 4C) and protein levels (Figure 4D), while the gene (Figure 4E) and protein (Figure 4F) expression of hnRNPA2/B1 were not affected by Cbx3 overexpression. These data strongly suggest that Cbx3 is regulated by hnRNPA2/B1.

hnRNPA2/B1 induces SMC differentiation, at least in part, through regulation of Cbx3 gene expression.

Next, we wondered if Cbx3 plays a role in hnRNPA2/B1-mediated SMC differentiation. Knockdown of Cbx3 with specific siRNA significantly abrogated the expression of SMC differentiation genes induced by hnRNPA2/B1 overexpression (Figure 5A), suggesting that Cbx3 is crucial for hnRNPA2/B1-mediated SMC differentiation. To confirm that Cbx3 is essential in hnRNPA2/B1-mediated SMC gene expression, day 3 differentiating ES cells were co-transfected with control (pCMV5) or Cbx3 expression plasmids pCMV5-Cbx3 (2µg/10^6 cells) and control random siRNA or hnRNP A2/B1siRNAs (100nM). Data shown in Figure 5B demonstrated that knockdown of hnRNPA2/B1 significantly downregulated SMαA and SM-MHC gene expression, while overexpression of Cbx3 dramatically upregulated the expression of these genes. Importantly, compared to hnRNPA2/B1 siRNA transfection alone, co-transfection of hnRNPA2/B1 siRNAs and Cbx3 expression plasmid significantly increased SMαA and SM-MHC gene expression. This suggests that Cbx3 overexpression can rescue the inhibitory effects of SMC differentiation gene expression mediated by hnRNPA2/B1 siRNA. Taken together, these data strongly indicate that apart from the transcriptional regulation of SMC gene expression, hnRNPA2/B1 can also regulate Cbx3 to mediate SMC differentiation.
hnRNPA2/B1 knockdown in neural crest cells causes maldevelopment of chick embryos

To explore the in vivo relevance of hnRNPA2/B1 in SMC differentiation and cardiovascular development, whole mount immunohistochemical staining with hnRNPA2/B1 antibody and whole mount in situ hybridization staining with digoxin-labeled antisense RNA probe specific for hnRNPA2/B1 were performed on chick embryos as described previously(7). We observed a consistent positive staining on the developing heart and the first four branchial arch arteries (data not shown). Importantly, hnRNPA2/B1 expression colocalized with SMαA in the floor of dorsal aorta as demonstrated by triple-immunofluorescent staining (Figure S1), suggesting that hnRNPA2/B1 might be involved in vascular smooth muscle development in the chick embryo.

To examine the involvement of hnRNPA2/B1 in the differentiation of neural crest cells into SMCs, specifically designed hnRNPA2/B1 or control morpholinos (Gene Tools, LLC) were introduced into the right-hand side of the neural crest using electroporation. The electroporator was set at 7.5 volts, 4 pulses, length of 50ms and interval of 1000ms as previously described(7). Twenty-four hours after transfection, embryos were checked and only those with the correct fluorescence labeling (hnRNPA2/B1 morpholino v.s control morpholino, n=12 v.s 8, respectively) were included for further evaluation. Whole mount immunohistochemistry showed significant inhibition of hnRNPA2/B1 protein expression in neural tubes that had been transfected with hnRNPA2/B1 morpholino, while the expression of hnRNPA2/B1 in neural tube electroporated with control morpholino was not affected (Figure S2). Importantly, we observed that chick embryos electroporated with hnRNPA2/B1 morpholino grew slowly, and most of them were smaller than embryos in the control group. Particularly, 48 hours after electroporation, branchial arch arteries in the hnRNPA2/B1 morpholino chick embryos did not seem to function well, with either nil or reduced blood circulation. In comparison, no observed embryos in the control morpholino group developed abnormally, even at a later stage (Figure 6A, B). Chi-Square Tests showed that the percentage of embryos with maldevelopment in hnRNPA2/B1 morpholino group was dramatically higher than that of control morpholino group (p<0.0001, Figure 6C).

hnRNPA2/B1 knockdown inhibits neural crest cell migration and differentiation

To investigate the underlying mechanisms in the maldevelopment or death of embryos, five embryos from each group were selected randomly to perform double immunofluorescent staining for SMαA and fluorescein expression to trace SMC differentiation and migration of neural crest cells in the developing chick embryos. Our data revealed that the branchial arch arteries smooth muscle, particularly the 3rd and 4th on
the right-hand side of the embryos in the hnRNPA2/B1 morpholino group did not develop completely (Figure 7BR, red arrow; Figure S3). However, branchial arch arteries at the left side of the hnRNPA2/B1 morpholino group and both sides of embryos in control morpholino group (Figure 7AL, AR and BL; Figure S3) developed normally. Such developmental defects could result from either the delayed migration of neural crest cells transfected with hnRNPA2/B1 morpholino (indicated by yellow arrows in Figure 7BR; Figure S3), or differentiation of neural crest cells into SMCs was abolished if migration is not affected (indicated by white arrows in Figure 7BR; Figure S3). In comparison, neural crest cells transfected with control morpholino not only migrate into vessel wall but also differentiate into SMαA positive cells (white arrows in Figure 7AR; Figure S3). These data strongly suggest that the knockdown of hnRNPA2/B1 in neural crest cells delays the cell migration and inhibits SMC differentiation, resulting in developmental defects of branchial arch arteries of chick embryos. Subsequently, these lead to the breakage of arteries (red arrows in Figure 7B, BR; Figure S3) and lethality of embryos.

Discussion

Adult SMCs retain significant plasticity in order to carry out different functions. After vascular injury and in response to local inflammation factors or cytokine stimulations, vascular contractile SMCs undergo phenotype switching to acquire either synthetic or dedifferentiated phenotype. These SMCs then participate in the formation of neointima by decreasing the expression of SMC differentiation markers(1). Similarly, during the progression of atherosclerosis, recruited SMCs also acquired a transition to a synthetic phenotype in lesion formation(30). Studies on the mechanism of SMC de/differentiation are not only crucial for exploring human pathologies but also useful for isolating and differentiating stem cells or vascular progenitors to SMCs, which is required for tissue-engineered vessel. In the present study, we uncovered important roles of nuclear protein hnRNPA2/B1 in SMC differentiation from ES cell and chick embryo arteriogenesis. Our current data strongly suggest that hnRNPA2/B1 is a potential modulating target to efficiently derive SMCs from stem cells for cardiovascular regenerative medicine.

hnRNPA2/B1 and SMC differentiation

The hnRNPA2/B1 gene gives rise to two different isoforms, A2 and B1. B1 mRNA is alternatively spliced from the primary hnRNPA2/B1 gene transcript by including a 36-bp exon, which adds 12 amino acids near the amino terminus in B1 isoform(15). Kamma et al(31) showed that hnRNPA2/B1 is localized in the nucleus with considerable tissue-specific variation in rat. This is consistent with the immunofluorescent staining that we performed in differentiating SMCs, where hnRNPA2/B1 localized in the nucleus.
hnRNPA2/B1 is involved in various different nuclear activities, such as post-transcriptional regulation of RNA splicing and transportation, or transcriptional regulation of c-myc, apolipoprotein E (apo E), vitamin receptor gene, breast cancer 1 (BRCA1) and gonadotropin-releasing-hormone 1 (GnRH1)(22-26). Most hnRNPA2/B1 studies were carried out to study cancer cell metabolism and phenotype switch, which are related to alternative splicing(32-37). However, little is known about hnRNPA2/B1 in stem cell differentiation, especially in SMC lineage and embryonic vascular development.

At the start of the study, hnRNPA2/B1 was revealed using nuclear proteomics as one of several nuclear proteins whose protein levels were significantly up-regulated during early SMC differentiation from ES cell (data not shown), which strongly implies that hnRNPA2/B1 may play a role in SMC differentiation.

Subsequently, the importance of hnRNPA2/B1 in SMC differentiation from ES cell was further demonstrated using gain and loss of hnRNPA2/B1 gene functions in differentiating ES cells. We have demonstrated that enhanced hnRNPA2/B1 gene expression significantly promotes SMC differentiation, while knockdown of endogenous hnRBP2/A2/B1 gene expression dramatically inhibits SMC differentiation. No such effects were observed in differentiating ES cells on other cell lineage gene expression such as markers from endothelial cell (Flt-1 and CD144), haematopoietic progenitor cells (CD133 and Alcam), cardiac fibroblast (CD90 and Ddr2), cardiomyocyte (Actc-1 and Tnn-1), trophoblast (Dlx3 and Tpbg), mesenchymal stem cell (CD29 and CD44) and neural progenitors (Nestin and Gap43) (data not shown). Taken together, our findings indicate that hnRNPA2/B1 plays a critical role in mediating SMC differentiation from ES cells.

hnRNPA2/B1 regulates SMCs differentiation through a transcriptional mechanism

Although evidence showed that hnRNPA2/B1 regulates gene expression mainly via posttranscriptional modification and transport of pre-RNA15, 16, recent research also strongly suggests that hnRNPA2/B1 can regulate gene expression via a transcriptional mechanism. For instance, studies showed that hnRNPA2/B1 regulates c-myc, apo E, vitamin D receptor, BRCA1 and GnRH1 gene expression by binding to related gene promoters22-26. hnRNPA2/B1 has been reported as a gene transcriptional activator or repressor in different gene regulation. For example, hnRNPA2 transcriptionally repress expression of vitamin D receptor, but it works more like an activator for BRCA1 gene transcription(14,38). In this study, our current findings also revealed that hnRNPA2/B1 is an activator that regulates SMC SMαA and SM22 gene expression during SMC differentiation from ES cells. Although G-quadruplex formation in the promoter region and protein-protein interaction was proposed in hnRNPA2/B1 gene regulation(39,40), the
mechanism of hnRNPA2/B1 in transcriptional regulation remains unclear. Moreover, whether other functions of hnRNPA2/B1 such as alternative splicing and nuclear-cytosome transportation of mRNA are involved in SMC differentiation remains to be elucidated.

**hnRNPA2/B1 and Cbx3 interaction in SMC differentiation**

We have identified nuclear protein hnRNPA2/B1 as a critical SMC differentiation regulator in this study. Also, we previously reported that nuclear protein Cbx3 plays a crucial role in SMC differentiation from stem cells and in chick embryonic arteriogenesis(7). Interestingly, evidence has revealed a close link between hnRNPA2/B1 and Cbx3 gene(29). All this evidence has prompted us to investigate the functional interactions of hnRNPA2/B1 and Cbx3 and its significance in SMC differentiation from stem cells. Our data revealed that hnRNPA2/B1 is an upstream regulator of Cbx3. By applying expression plasmids and siRNAs co-transfection strategies, we further demonstrated that Cbx3 is required for hnRNPA2/B1-mediated SMC differentiation. These data strongly indicate that apart from direct activation of SMC gene transcription, hnRNPA2/B1 also regulates Cbx3 to promote SMC differentiation.

**hnRNP A2/B1 is involved in embryonic arteriogenesis**

hnRNP A2/B1 is expressed in multi-organogenesis such as neural, gastrointestinal, skeletal and cardiovascular system during early embryonic development. We found that hnRNPA2/B1 was strongly expressed in the dorsal aorta and branchial arches arteries, and colocalized with SMC specific marker SMαA in the floor of dorsal aorta in chick embryos. SMαA positive cells in HH 16 chick embryo were restricted to the aortic floor, where the first smooth muscle starts to form during embryonic aorta angiogenesis(41). Therefore, we speculated that hnRNPA2/B1 may play a role in the earlier induction of SMC differentiation during embryonic arteriogenesis. To address this possibility, instead of using siRNAs, electroporation of morpholino oligonucleotides on the neural crest in chick embryos was applied.

Firstly, although specific knockdown strategies using morpholino oligonucleotides or siRNA have been successfully proven to be powerful tools to study gene functions in different organisms in vivo, the off-target effects independent to siRNA sequence occasionally cause embryo developmental defects or even death(42,43). Whereas no such side effects was reported with morpholino, the use of morpholino oligonucleotides to inhibit protein translation of certain genes was hence a preferable approach for knockdown experiment during chick development(44).

Secondly, neural crest cells residing at the levels of rhombomere 2, 4, 6 and 7 of chick embryos at stages HH10-/HH10/HH10+ were chosen to perform in vivo electroporation using hnRNPA2/B1 morpholino
oligonucleotides. It is well known that neural crest cells are the only cell lineage contributing to smooth muscle during branchial arch angiogenesis\(^\text{(45,46)}\). In particular, neural crest cells residing at the level of rhombomere 2, 4, 6 and 7 will migrate into bronchial arch 1, 2, 3 and 4 and differentiate towards SMCs at day 3 and 4 in chick embryonic development\(^\text{(47-49)}\). Therefore, these neural crest cells serve as a specific target for studying the differentiation of embryonic progenitors into SMCs and the functional importance of any genes in branchial arch arteries development. As expected, stainings on cryosections clearly showed that the smooth muscle of the 3\(^{\text{rd}}\) and 4\(^{\text{th}}\) branchial arch arteries at the right-hand side of embryos transfected with hnRNPA2/B1 morpholino did not form completely. However, branchial arch arteries at the left-hand side and both sides of the embryos in control morpholino group, developed normally with at least the formation of a single layer of smooth muscle cells. Thus, the inhibition of hnRNPA2/B1 is responsible for the developmental defects of these branchial arteries in chick embryos, and eventually leading to embryo death.

**Summary**

The functional role of hnRNP A2/B1 in SMC differentiation from stem cells and embryonic branchial arteriogenesis has been demonstrated in the current study. We showed that hnRNPA2/B1 mediates SMC differentiation by direct binding and activates the promoters of SMC differentiation genes (SM\(\alpha\)A and SM22\(\alpha\)). We further identified a regulatory role of hnRNPA2/B1 on Cbx3 gene expression during SMC differentiation from stem cells. Our studies highlight the importance of hnRNPA2/B1 in SMC differentiation from both stem cells and chick embryonic arteriogenesis. These findings will significantly contribute to the understanding of the mechanisms in embryonic arteriogenesis and benefit future application in engineering tissue vessels.
Acknowledgments

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Reference

Figure Legends

**Figure 1. hnRNPA2/B1 is upregulated during SMC differentiation.**

(A) hnRNPA2/B1 mRNA was up-regulated during SMC differentiation. Undifferentiated ES cells were plated onto dishes coated with 5μg/ml of collagen IV and cultured in differentiation medium (DM). Total RNA from undifferentiated ES cells (d0) or differentiating ES cells at day 3 (d3), 5 (d5) and 7 (d7) were harvested and subjected to RT-qPCR analysis with primers specific for hnRNPA2/B1. (B) Nuclear protein from undifferentiated ESCs (d0) or differentiating ES cells at the indicated time points were harvested and subjected to Western blot analysis. Up panels show the representative images, and bottom panel show bar graphs representing means±SEM of densitometric analysis (n=3) of the relative protein levels of hnRNPA2B1. (C) Nuclear localization of hnRNPA2/B1 in differentiating ES cells. Immunofluorescence staining was conducted on day 7 differentiating ES cells with antibodies against hnRNPA2/B1. The data presented here are representative or an average of three independent experiments. Significant difference from control, *P<0.05.

**Figure 2. hnRNPA2/B1 is critical for SMC differentiation from ES cells.**

(A-C) hnRNPA2/B1 knockdown downregulated SMC-specific transcription factors and SMC markers. Two sets of hnRNPA2/B1-specific small interfering RNAs (hnRNPA2B1si-1 & -2) and random siRNA control were transfected into day 3 differentiating ES cells; after an additional 2-3 days of culture, total RNA and protein were harvested and subjected to RT-qPCR (A) and Western blot analyses (B-C). Panel B shows the representative images, and panel C show bar graphs representing means±SEM of densitometric analysis (n=3) of the relative protein levels of target proteins. (D-F) hnRNPA2/B1 overexpression enhanced collagen IV-induced SMC differentiation. Undifferentiated ES cells were nucleofected by nucleofector II with different amounts of hnRNPA2/B1 expression plasmids pCMV5-hnRNPA2/B1. Nucleofected cells were plated on dishes coated with 5 μg/ml of collagen IV and cultured for 3-4 days in DM. Total RNA and protein were harvested and subjected to RT-qPCR analysis for SMC gene expression (D), transcription factors (F) and Western blot analysis (E). An appropriate amount of empty vector pCMV5 was included as plasmid amount compensation. Same amount of protein was loaded into each well, blotted and probed with specific antibodies for HA (represents exogenous hnRNPA2/B1), SMαA, SRF, MEF2c and SM-MHC. α-tubulin was included as internal control (B and D). *p<0.05 vs. control. The data presented here were representative or an average of three independent experiments. *p<0.05 vs. control.
Figure 3. hnRNPA2/B1 transcriptionally activates SMC genes expression through direct binding to the promoters of SMC differentiation genes.

(A) Actinomycin D ablated hnRNPA2/B1 overexpression-mediated SMC gene expression. Day 3 differentiating ES cells transfected with pCMV5-hnRNPA2/B1 or pCMV5 (2.0µg/1×10⁶ cells) were treated with or without actinomycin D (1µg/ml) for 6 hours and harvested for RT-qPCR analysis of SMαA and SM22α gene expressions. DMSO was included as a control. The data presented here is an average of three independent experiments. *P<0.05 (2nd column vs. 1st column), **P<0.05 (4th column vs. 2nd column). (B) hnRNPA2/B1 regulated the promoter activities of SMC differentiation genes. ES cells were cultured on collagen IV and transfected at day 3 with luciferase reporter plasmids pGL3-SMαA-Luc (0.3µg/5×10⁴ cells) together with pCMV5-hnRNPA2/B1 or pCMV5 (0.4µg/5×10⁴ cells). pGL3-Renilla (0.05µg/5×10⁴ cells) was included as luciferase plasmid control. Luciferase and Renilla activity assays were detected 48 hours after transfection. Luciferase activity was normalised or defined as the ratio of luciferase activity to Renilla activity. The data presented here are an average of three independent experiments. *P<0.05 (vs. control). (C) hnRNPA2/B1 bound directly to the promoter regions within SMC differentiation genes. ChIP assays were performed using antibodies against hnRNPA2/B1 and analyzed as described before. The data presented here are an average of three independent experiments. *P<0.05 (vs. control).

Figure 4. hnRNPA2/B1 regulates Cbx3 gene expression.

(A and B) Knockdown of hnRNPA2/B1 downregulated Cbx3 gene expression. Cbx3-hnRNPA2/B1-specific siRNAs and random siRNA control were transfected into day 3 differentiating cells; after an additional 48 hours or 72 hours of culture, total RNA and protein were harvested and subjected to RT-qPCR (A) and Western blot analysis (B). α-tubulin was included as internal control. (C and D) hnRNPA2/B1 gene expression was not affected by Cbx3 overexpression. (E and F) hnRNPA2/B1 overexpression significantly increased Cbx3 gene and protein expression levels. Undifferentiated ES cells were nucleofected using nucleofector II with different amounts of hnRNPA2/B1 expression plasmids pCMV5-hnRNPA2/B1. Nucleofected cells were plated on dishes coated with 5 µg/ml of collagen IV and cultured for 3-4 days in DM. Total RNA and protein were harvested and subjected to RT-qPCR (E) and Western blot analyses (F). Appropriate amounts of empty vector pCMV5 (control) were included to ensure total DNA concentrations for all experiments were equal. The data presented here are representative or mean±SEM of three independent experiments, *P< 0.05. Numbers under the bands in the panels B, D and F
indicate the average relative expression levels of target proteins from three independent experiments.

**Figure 5. hnRNPA2/B1 mediates SMC differentiation partially through regulation of Cbx3 gene expression.**

(A) Cbx3 gene activation was essential for hnRNPA2/B1-mediated SMC gene expression. Day 3 differentiating ES cells were cotransfected with control (pCMV5) or hnRNPA2/B1 expression plasmids pCMV5-hnRNPA2/B1 (2μg/10^6 cells) and control random siRNA or Cbx3 siRNAs (100nM). Total RNA were harvested 72 hours after transfection and subjected to RT-qPCR analysis. (B) Impairment of SMC genes expression mediated by hnRNPA2/B1 knockdown was rescued by Cbx3 overexpression. Day 3 differentiating ES cells were cotransfected with control random siRNA or hnRNPA2/B1 siRNAs (100nM) and control (pCMV5) or Cbx3 expression plasmids pCMV5-Cbx3 (2μg/10^6 cells). Total RNA was harvested 72 hours after transfection and subjected to RT-qPCR analysis. The data presented here are a representative or mean±S.E.M. of three independent experiments, *P< 0.05.

**Figure 6. Inhibition of hnRNPA2/B1 causes maldevelopment of chick embryos.**

(A and B) Representative images of embryos transfected with hnRNPA2/B1 and control morpholino oligonucleotides. Most embryos in hnRNPA2/B1 morpholino group have reduced blood circulation in branchial arch arteries (B) in comparison with embryos electroporated with control morpholino (A). Black arrow indicates branchial arch arteries. (C) Maldevelopment of chick embryos were caused by inhibition of hnRNPA2/B1 expression in NCCs. Chi-Square analysis was performed using SPSS 17.0 software. It was that found hnRNPA2/B1 morpholino significantly affected the chick embryonic development (p<0.0001). Scale bar represents 1mm.

**Figure 7. Inhibition of hnRNPA2/B1 in NCCs results in developmental defects of 3rd and 4th branchial arch arteries**

(A and B) Triple stainings for DAPI (blue), SMαA (green) and morpholino oligonucleotides (red fluorescein) with sections from embryos electroporated with control (A) or hnRNPA2/B1 (B) morpholino oligonucleotides. In hnRNPA2/B1 morpholino group, fluorescein-labeled NCCs could not migrate into branchial arch arteries at the right-hand side (Figure 7BR, yellow arrows) and differentiate into SMCs (Figure 7BR, white arrows), leading to the developmental defect of smooth muscle in the 3rd and 4th branchial arches (Figure 7BR, indicated by red arrows). In contrast, the left-hand side of branchial arch arteries (Figure 7BL) developed normally. In control morpholino group, both sides of
branchial arch arteries showed complete smooth muscle formation (Figure 7AR and AL). The fluorescein-labeled NCCs could not only migrate into branchial arch arteries wall at the right-hand side, but also differentiate into SMCs in the 4th branchial arch artery. White arrows indicate those NCCs which are differentiating to SMCs. Representative images from five embryos in each group are presented here. Scale bar represents 50μm. Isotype IgG substituted primary antibody as negative control during staining process. iii, 3rd branchial arch artery; iv, 4th branchial arch artery. Original un-merged images of Figure 7AR and 7BR were provided in online supplemental data (Figure S3).
Figure 1

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A

hnRNPA2/B1 mRNA relative level

0 3 5 7 days

B

hnRNPA2/B1 total protein relative level

0 3 5 7 days

C

hnRNPA2/B1

DAPI

Merge

10μm
Figure 2

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A

B

C

D

E

F

- Reagent only
- hnRNPA2/B1 siRNA-1
- Control siRNA
- hnRNPA2/B1 siRNA-2

mRNA relative level

pCMV5 (µg)              2.0           1.5            1.0             0.0
phnRNPA2/B1 (µg)  0.0           0.5            1.0              2.0

* * *

SRF
Myocardin
MEF2c

E

HA/hnRNPA2/B1
SMαA
SM-MHC
SRF
MEF2c
α-tubulin

pCMV5 (µg) phnRNPA2/B1 (µg) 2.0 1.5 1.0 0.0

0.20 0.40 0.60 0.80 1.00

Protein relative level

pCMV5 (µg) phnRNPA2/B1 (µg) 2.0 1.5 1.0 0.0

0.20 0.40 0.60 0.80 1.00

mRNA relative level

pCMV5 (µg) phnRNPA2/B1 (µg) 2.0 1.5 1.0 0.0

0.20 0.40 0.60 0.80 1.00
Figure 6

C hnrnpA2/B1 morpholino affects chick embryonic development

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Functional impact of heterogeneous nuclear ribonucleoprotein A2/B1 in smooth muscle differentiation from stem cells and embryonic arteriogenesis
Gang Wang, Qingzhong Xiao, Zhenling Luo, Shu Ye and Qingbo Xu

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