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Original Article

Evidence for AJUBA-catenin-CDH4-linked differentiation resistance of mesenchymal stem cells implies tumorigenesis and progression of head and neck squamous cell carcinoma: a single-cell transcriptome approach

Andres Stucky[1], Li Gao[1], Lan Sun[1], Shengwen Calvin Li[1,2,3*], Xuelian Chen[1], Tiffany H. Park[1], Jin Cai[1], Mustafa H. Kabeer[4,5], Xi Zhang[1], Uttam K. Sinha[1], Jiang F. Zhong[1*]

[1] *Department of Otolaryngology, Keck School of Medicine, University of Southern California, Los Angeles, CA 90033, USA;*
[2] *Neuro-Oncology and Stem Cell Research Laboratory, Center for Neuroscience Research, CHOC Children's Research Institute, Children's Hospital of Orange County (CHOC), Orange, CA 92868, USA;*
[3] *Department of Neurology, University of California - Irvine School of Medicine, Orange, CA 92868, USA;*
[4] *Division of Pediatric General and Thoracic Surgery, Children's Hospital of Orange County, Orange, CA 92868, USA;*
[5] *Department of Surgery, University of California - Irvine School of Medicine, Orange, CA 92868, USA.*

ABSTRACT

An increasing number of reports indicate that mesenchymal stem cells (MSCs) play an essential role in promoting tumorigenesis and progression of head and neck squamous cell carcinoma (HNSCC). However, the molecular mechanisms underlying this process remain unclear. Using the MSC model system, this study analyzes the molecular pathway by which differentiation resistant MSCs promote HNSCC. MSCs were cultured in osteogenic differentiation media and harvested on days 12 and 19. Cells were stained for cell differentiation analysis using Alizarin Red. The osteogenesis-resistant MSCs (OR-MSCs) and MSC-differentiation-derived osteoblasts (D-OSTBs) were identified and subjected to the single-cell transcriptome analysis. Gene-specific analyses of these two sub-populations were performed for the patterns of differential expression. A total of 1 780 differentially expressed genes were determined to distinguish OR-MSCs significantly from D-OSTB. Notably, AJUBA, β-catenin, and CDH4 expression levels were upregulated considerably within the OR-MSCs compared to D-OSTBs. To confirm their clinical relevance, a survey of a clinical cohort revealed a high correlation among the expression levels of AJUBA, β-catenin and CDH4. The results shed new light that OR-MSCs participate in the development of HNSCC *via* a pathway mediated by AJUBA, β-catenin, CDH4, and CTNNB1, thereby implying that MSC-based therapy is a promising therapeutic approach in the management of HNSCC.

Keywords: mesenchymal stem cells, AJUBA, β-catenin, CDH4, head and neck squamous cell carcinoma, differentiation resistance, tumorigenesis, cancer progression, CTNNB1

[*]Corresponding to: Shengwen Calvin Li, CHOC Children's Research Institute, Orange, CA 92868, USA. E-mail: shengwel@uci.edu; Jiang F. Zhong, Department of Otolaryngology, Keck School of Medicine, University of Southern California, Los Angeles, CA 90033, USA. E-mail: jzhong@usc.edu.

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INTRODUCTION

Cancers of the head and neck are the sixth most commonly diagnosed cancers worldwide[1]. Squamous cell carcinoma accounts for more than 630 000 cancer cases and 350 000 annual deaths globally[2–3]. In the United States alone, head and neck cancer constitutes about 3% of total malignancies, with approximately 53 000 Americans developing head and neck cancer annually and 10 800 dying from the disease[4]. The future societal burden will likely be even higher due to the increasing prevalence of risk factors such as smoking, alcohol consumption, unhealthy diet, and human papillomavirus (HPV) infection. Mesenchymal stem cells (MSCs) have shown great potential for use in regenerative medicine but display chemoattractant properties towards tumor sites and are found in much greater numbers in head and neck squamous cell carcinoma (HNSCC) when compared to healthy tissue[5]. Intensive research in cancer biology reveals a unique mode of the interplay between tumor cells and MSCs. MSCs serve as intermediators among tumor cells *via* their network that, in turn, contribute to the progression of cancer. The MSC-associated chemoattractant property of HNSCC raises the concern that migration of these cells towards tumor sites could aid in HNSCC's progression, as the malignant transformation of MSCs has been reported in various studies, including cell fusion of gastric epithelial cells with MSCs for epithelial-to-mesenchymal transition-based malignancy[6], and snail and HNF4alpha-mediated MSC malignancy in liver cancer[7].

MSCs are a vital component of the bone marrow that show the capacity to self-renew and differentiate in culture into mesodermal-derived tissues, such as chondrocytes, adipocytes, myoblasts, osteoblasts, and hematopoietic cells[8–9]. MSCs can migrate to specific organs and tissues[10–12]. However, there is increasing evidence that there is a subpopulation of differentiation-resistant MSCs recruited to sites of tissue damage, which can play an important role in tumorigenesis[13]. This subset of osteogenesis-resistant MSCs (OR-MSCs) can lead to cancer upon activation of local chronic inflammation signals *in vivo*[11] and is thus considered a cancer stem cell (CSC)-like population[12]. Cancers formed by these OR-MSCs are similar to some tumors initiated by normal epithelial cells by the convergence of malignant reprogramming[14]. Therefore, we investigated the variation among MSCs during the process of osteogenic development and identified a subpopulation of OR-MSCs *via* single-cell transcriptome analysis of differentiating MSCs[15]. This approach is based on findings of our previous reports that single-cell transcriptomes can help map out a specific pathway for cancer relapse[16] and determine the mechanisms of action for cancer biomarkers[17]. Thus, the subpopulation of osteogenesis-resistant MSCs can be identified by single-cell transcriptome-based dendrograms that cluster individual cells together by their gene expression similarity.

Despite the various efforts aimed at understanding differentiation resistant MSCs, the molecular mechanism underlying MSCs' resistance to differentiation remains unclear. This report investigates the Lim domain-containing protein ajuba (AJUBA) as a critical component in head and neck squamous cell carcinoma (HNSCC) carcinogenesis osteogenesis inhibition and maintenance CSC characteristics of MSCs. The Zyxin/AJUBA family of proteins is characterized by a tandem LIM motif in their C-termini. AJUBA interacts with various adhesion proteins, Hippo and Wnt effector proteins to form complexes that exert biological functions, such as cell adhesion, mitosis, and apoptosis. Recently, studies have identified AJUBA as an essential regulator of progression in various cancers[18–20]. AJUBA also plays an integral role in the oncogenic process of epithelial to mesenchymal transformation[21–22].

Increasing studies show that AJUBA exerts an effect as an oncogene or onco-suppressor, depending on the cellular context, through its interactions with essential components of the Hippo signal transduction pathways. On the one hand, AJUBA can negatively regulate Hippo signaling by inhibiting YAP's phosphorylation, thereby preventing transcription of its target genes in hepatocellular carcinoma[23]. On the other hand, AJUBA is also known to promote HNSCC[20], possibly through deregulation of Hippo pathway activity. Our results indicate that OR-MSCs show significant enrichment of the Hippo pathway, associated proteins, and identify the Hippo pathway regulator, AJUBA, as a key upregulated feature in osteogenesis resistant single mesenchymal stem cells.

Mechanistically, it has been reported that AJUBA binds β-catenin to negatively regulate the Wnt signaling pathway by promoting GSK-3β-mediated phosphorylation of β-catenin[24]. β-Catenin is a core component of Wnt canonical signaling pathways and has emerged as a critical player in head and neck squamous cell carcinoma (HNSCC)[25–27]. β-Catenin/Wnt-mediated signaling contributes to the advancement of oral squamous cell carcinoma (OSCC) and resistance to current therapies[28–29]. β-Catenin also regulates the proliferation and self-renewal of cancer stem cells (CSCs). The CDH4 gene

is located on chromosome 20q13.3 and encodes the R-cadherin protein[9]. R-cadherin is a classic cadherin. The highly conserved transmembrane adhesion, calcium-dependent glycoproteins regulate cell growth, mobility, and differentiation. CDH4 is also known along with vimentin and snail to be a cellular marker for epithelial to mesenchymal transformation[30]. Therefore, we sought to explore the relationship among AJUBA, β-catenin, and CDH4 in MSCs and HNSCC to analyze their relationship.

MATERIALS AND METHODS

Normal, human bone marrow-derived mesenchymal stem cells were obtained from ATCC (ATCC® PCS-500-012™). All cells were expanded using low-glucose Dulbecco's Modified Eagle Medium (DMEM, Corning), supplemented with 10% PBS and 1% penicillin streptavidin (GIBCO). Cells were genotyped to confirm identity using a PCR-based assay for positive MSC markers CD10, CD13, CD29, CD73 and CD44, negative for CD14, CD34, CD19, and CD45. Mycoplasma contamination was assessed with Hoechst 33 258 staining under a high-magnification fluorescent microscope. Once cells reached 100% confluence, low-glucose DMEM was replaced by StemPro™ Osteogenesis Differentiation Medium (Thermo Fisher Scientific, Canoga Park, CA), matched with a control group of unmanipulated MSCs cultured in low-glucose DMEM. All cells (OR-MSC and D-OSTB) were harvested after 12 and 19 days from the osteogenic medium. Differentiating MSC gene expression was assessed at both 12- and 19-day time points.

Alizarin red staining

MSCs were first fixed in a buffer containing 10% formalin and incubated for 1hr. Cells were washed three times with PBS, after which they were incubated in a 2% W/V solution of Alizarin Red and kept at room temperature in the dark for 30 minutes. Following three more washes with Alizarin Red, staining was visualized under a Nikon Eclipse TE300 inverted microscope at 4X amplification. The intensity was quantified using ImageJ (NIH). Images were converted to grayscale, and the relative numbers of differentiated cells were measured by counting the ratio of alizarin-stained cells on the culture plate to the total area of cultured cells.

Single-cell capture

All MSCs were detached from the plates by incubation with 100 microliters of trypsin for 5 minutes at 37 °C, and an additional 1 mL of culture medium was used to terminate the trypsin reaction. Following 3 washes in PBS, cells were resuspended in 1 mL of PBS and injected into a pneumatic-gated single-cell microfluidic capturing chip. The capture of an individual single cell in the isolated microfluidic chambers was visually confirmed at 4X amplification using real-time imaging under a Nikon Eclipse TE300 inverted microscope. A total of 18 cells were collected, and following total RNA quantification for quality control, 5 cells were selected for each condition.

RNA extraction and library preparation

TRIzol® reagent (Life Technologies) was used to isolate messenger RNA. RNA was processed using the REPLI-g WTA single-cell system (Qiagen). Amplified double-stranded cDNA was fragmented using NEB double-stranded DNA fragments. A screen tape system (Agilent) was used to quantify fragmented DNA for library prep input. A NEBNext Ultra Ⅱ DNA library prep kit for Illumina Barcoded libraries was used to process the 100 ng of fragmented cDNA and libraries prepared using an Illumina TruSeq Stranded mRNA library prep kit. The obtained libraries were submitted for RNA sequencing to the Loma Linda University Center for Genomics.

Single-cell RNA-seq and transcriptome analysis

Libraries were sequenced on the Illumina HiSeq 4 000 platform (Illumina). Adaptor contamination was trimmed, and low-quality and duplicated reads were removed using Trimomatic[31], where bases with Phred scores < 20 were excluded and reads shorter than 25 nt were removed from downstream analyses. On average, for every single cell, there were 2 million reads generated. Sequencing data were processed using Partek Flow v4 (Partek Inc.). Both pre- and post-alignment QA/QC was performed as part of the Partek Flow workflow. Reads were then aligned to human genome hg38[32] using Tophat 2.0.8 with default settings, using Gencode 20 as guiding annotation (www.gencodegenes.org). Gene reads were normalized by adding 0.01 divided by total counts, multiplied by ten thousand, and log-transformed.

Differential gene expression analysis

Exploratory analysis of gene expression was performed using principal component analysis (PCA) as part of the Partek Flow package, and two individual groups identified by PCA were selected for differential expression analysis using Partek's Gene

Specific Analysis (excluding genes with less than 10 reads per cell). Significantly differentially expressed genes were selected using a false discovery rate (FDR) cutoff adjusted to $P<0.05$ (Poisson regression). Ingenuity Pathway Analysis (IPA) software (Qiagen Bioinformatics) was used to identify the most prominent biological signaling pathways that differentiated the clusters. Molecules associated with pathways with the lowest *P*-values were chosen for clinical meta-analysis.

Clinical data

Using the keyword "AJUBA", we queried the web-based genomic analysis interactive tool, cBioPortal[33–34], (https://www.cbioportal.org), to survey all the available datasets curated by The Cancer Genome Atlas (TCGA), for differential gene expression between healthy and cancer patients, as well genetic alteration prevalent within the coding regions AJUBA in the same cohorts. Raw data for all cohorts can be found at the TCGA Genomic Data Commons portal (https://portal.gdc.cancer.gov). We used ONCOMINE[35] (https://www.oncomine.org), cancer microarray archive, and data mining tool to investigate additional datasets that showed AJUBA overexpression specifically in head and neck cancer tissue. Data were queried using the keywords "AJUBA" and "head and neck cancer *vs.* normal" to compare differential expression of head and neck cancer subtypes with their respective normal tissue. Only datasets showing significant differences ($P\leq0.05$) between normal and cancer tissue were incorporated.

RESULTS

Single-cell transcriptome analyses reveal genetic profiles of MSCs

Alizarin Red was first used as an indicator of bone mineralization that quantified calcium deposition, measured by colorimetric means. Following 12 days of incubation in differentiation media, 20% of the cells had differentiated. After 19 days in differentiation media, 80% of MSCs had differentiated (***Supplementary Fig. S1***) . Lysate aliquots from each group were harvested and processed *via* a pneumatic-gated microfluidic pump for single-cell analysis, as previously discussed[36]. All single cells were pooled for exploratory analysis of their transcriptomes using principal component analysis (PCA). Single cells clustered into two well-defined groups separated across the first two principal components with specific gene expression profiles associated with OR-MSCs and differentiated osteoblasts (D-OSTBs).

A total of 3 126 genes were identified with significantly upregulated expression and 117 genes were with significantly down-regulated expression in the D-OSTB group relative to the OR-MSC group. A total of 1 256 genes were significantly different among the two groups ($P\leq0.05$) with (>2 fold) different expression levels.

Gene expression profiles

Further investigation using a panel of cell markers for D-OSTBs indicated that a fraction of the cells from each harvested timepoint (12 and 19 days) had differentiated into mature osteoblasts while others had not. The gene expression comparison of the two groups revealed a pronounced up-regulation of osteogenic gene markers in D-OSTBs compared to OR-MSCs. D-OSTBs showed high transcription levels of osteogenic lineage markers RUNX2 (P=0.40), BMP4 (P=0.003 8), and BMP2 (P=0.00028) (***Supplementary Fig. S2***) and the most significant differences in the transcription levels of BMP6 (P=0.013), BMPR1B (P=0.001), and BMPR1A (P=1.64E-8) (***Fig. 1***) when compared to OR-MSCs. OR-MSCs showed significantly higher expression of pluripotent MSC markers vimentin (P=0.010) and CD73 (P=0.025) (***Supplementary Fig. S2***). Thus, our results confirmed that the D-OSTB group successfully differentiated into mature osteoblasts while the OR-MSC group remained undifferentiated.

Pathway analysis of differentiating MSCs

Guided by the PCA results, we then used the set of significantly differentially expressed genes between OR-MSCs and D-OSTBs in conducting a molecular enrichment analysis using Ingenuity Pathway Analysis (IPA®), intending to identify the most prominent molecular cascades differentiating OR-MSCs from D-OSTBs. The signaling mechanisms were identified, manifesting that most genes were involved in the cell cycle: G1/S checkpoint regulation (P=1.60E-4). The HIPPO signaling cascade (P=1.92E-05) was identified as the most meaningfully different pathway between OR-MSCs and D-OSTBs. We used this data to perform our analysis of the top differentially expressed genes between the two groups. This finding was consistent with increased gene expression of the cancer-associated stem cell marker AJUBA (P=0.03), the membrane-associated epithelial to mesenchymal transition (EMT) membrane protein CDH4 (P=0.008), and the catenin association proteins CTTNA1 (P=0.021) and CTNNB1 (P=0.014) (***Fig. 2***), depending on the

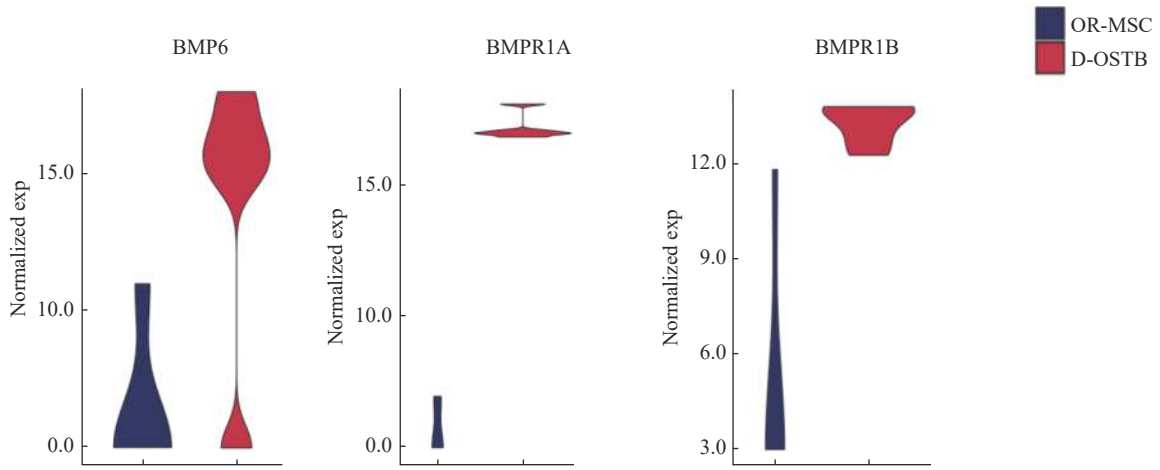

Fig. 1 **The normalized differential expression of bone morphogenic proteins between OR-MSCs and D-OSTBs: BMP6 (*P*=0.017), BMPR1A (*P*=2.1E-5), and BMPR1B (*P*=0.006)).** Y-axis represents averaged normalized values for the respective group OR-MSC (blue), D-OSTB (red).

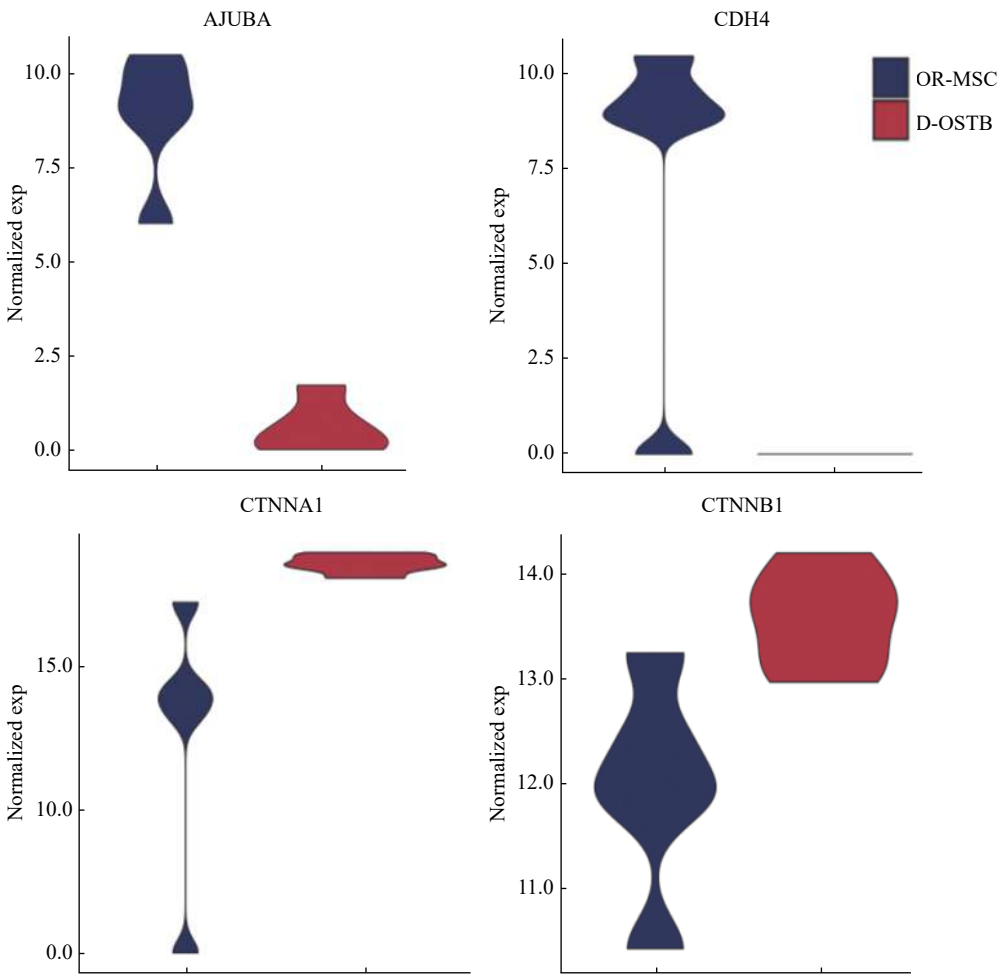

Fig. 2 **The normalized differential expression between OR-MSCs (blue) and D-OSTBs (red) for AJUBA (*P*=0.020), CDH4 (*P*=0.008), CTNNA1 (*P*=0.044) and CTNNB1 (*P*=0.050).** Y-axis represents averaged normalized values for the respective group OR-MSC (blue), D-OSTB (red).

environment can affect OR-MSCs' retention of stem cell characteristics.

The identified signaling mechanisms suggested most genes involved in the cell cycle: G1/S

checkpoint regulation (*P*=1.60E-4) and the HIPPO signaling cascade (*P*=1.92E-05) were identified as being the most meaningfully different pathways between OR-MSCs and D-OSTBs.

Clinical Data

To corroborate our single-cell results, we analyzed data from 32 types of cancer, including 1 084 patients from The Cancer Genome Project (TCGA), available in the cancer genomic analysis tool cBioPortal[33–34], which is an open-access resource for multidimensional cancer genomic data exploration. We confirmed that the Lim domain-containing protein AJUBA was consistently upregulated in various common cancers compared to normal tissues. AJUBA was found to be upregulated in 32 different types of cancer (***Fig. 3***). The highest expression values for AJUBA could be observed in head and neck, cervical and esophageal cancers (***Fig. 3***). AJUBA showed genetic alterations in 26 of the 32 analyzed types of cancer. Among these alterations, 18 types of cancers contained mutations, 20 cancers contained amplifications, 8 of them contained deep deletion (indicating, possibly a homozygous deletion), and two contained fusions in the coding region of AJUBA. Among all the surveyed cancers, head and neck cancer showed the highest rate of AJUBA mutation (***Fig. 4***)[33–34]. We further investigated additional datasets available from the Oncomine database[35] and confirmed in a cohort of 1 373 patients that AJUBA was overexpressed in five independent head and neck cancer studies[37–41].

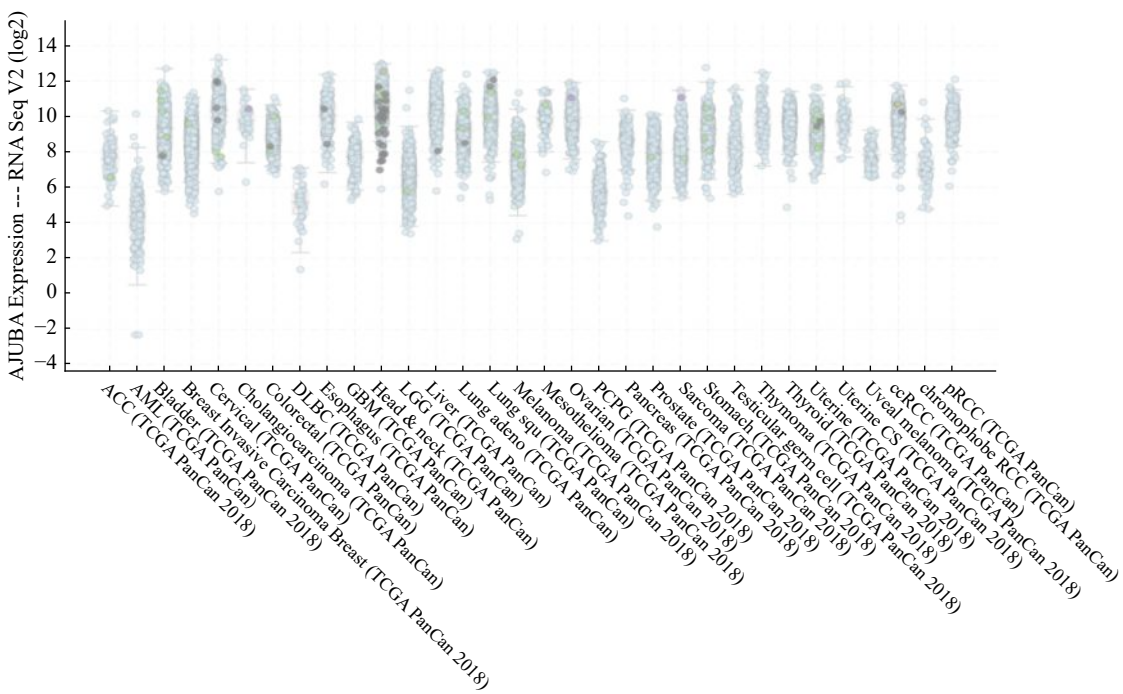

Fig. 3 **AJUBA normalized expression in 32 types of cancer available from TCGA PanCancer 2018 study.** Among all types of cancer, AJUBA was shown to be highest expression in HNSCC and cervical cancer (cBioPortal). Y-axis represents RSEM (Batch normalized from Illumina HiSeq_RNAseqV2) (log2 (value+1)).

DISCUSSION

In this study, we investigated single-cell transcriptomes of differentiating MSCs to evaluate heterogeneity in the MSC population. We were able to identify a subpopulation of these cells that retain their stem cell characteristics despite osteogenic stimulation and demonstrate a marked upregulation of AJUBA, CDH4, and CTNNB1. Our data are consistent with the literature that AJUBA is associated with epithelial-to-mesenchymal transformation in highly aggressive hepatocellular carcinoma phenotypes and colon cancer, as well as tumor metastasis in colon cancer[21]. AJUBA is known to inhibit the effect of retinoic acid[42], a compound that in culture causes embryonic carcinoma cells to differentiate into glial cells[43]. In a meta-analysis of 32 different types of cancer available at TCGA, HNSCC showed the highest AJUBA expression among all cancers and the highest mutation rate (***Fig. 4***). This finding could indicate that mutations in the AJUBA coding region could be enhancing its expression in head and neck

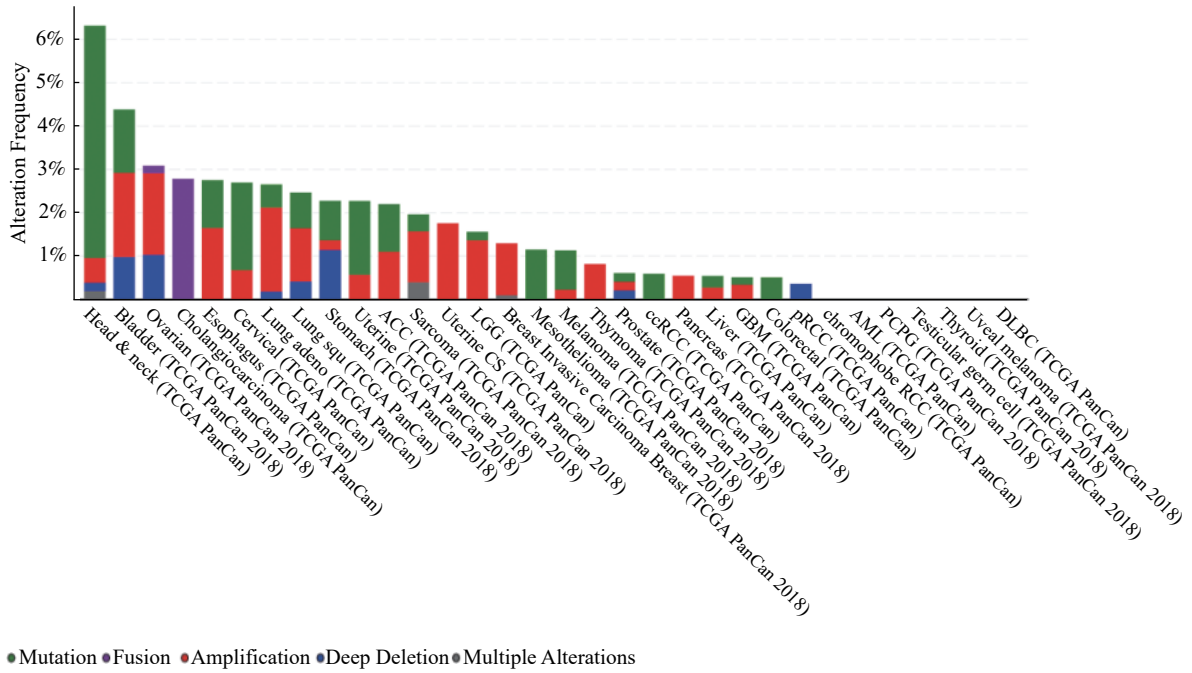

Fig. 4 **Relationship between AJUBA mutation frequencies and organ-based cancer types.** Among 32 types of cancers analyzed in cBioPortal, curated by The Cancer Genomic Atlas (TCGA) and available at the National Cancer Institute Genomic Data Commons (GDC), 18 contain mutations, 20 amplifications, 8 deep deletions, and 2 contain fusions in AJUBA. Head and neck cancers showed the highest mutation rate in AJUBA among all the surveyed cancers (cBioPortal). Y-axis represents percentage cases with AYUBA alterations for specific cancer indicated.

cancer, or alternatively, that high AJUBA expression levels and high gene mutation rates could have a synergistic effect in aggressive head and neck carcinomas.

Interestingly, both head and neck and cervical cancer, which showed the highest AJUBA expression among all cohorts (***Fig. 3***), have also been strongly associated with infection of HPV. AJUBA has also been extensively reported to be overexpressed in colon cancer and colon adenocarcinomas[44–46] as well as gastric[47–48], esophageal[49], and brain tumors[50–51]. AJUBA and CDH4 have been shown to play a role in EMT, however; studies on CDH4 in this context are scarce. Some heterogeneity has been observed during EMT studies comparing metastatic tissue with primary tumors in several types of cancers[52–55].

Up-regulation of AJUBA, CDH4, and CTNNB1 was further confirmed in a metanalysis of 32 types of cancer studies of patients with head and neck cancer. β-catenin-AJUBA cross-talk with cadherins controls transcription programs involved in cell proliferation, stemness, and differentiation. These programs are involved in mediating kinase cascade elements regulated by intrinsic and extrinsic signals, such as mechanical force, cell-cell contact, polarity, energy status, stress, and many diffusible hormonal factors. AJUBA and CDH4 might work cooperatively to antagonize Wnt/β-catenin in a HIPPO-independent manner or through the association of AJUBA with α-catenin[56–59]. These findings are in accordance with our results and suggest that a subpopulation of MSCs could be responsible for tumorigenesis after MSC transplantation. In our model, MSC tumorigenesis is likely mediated through a CDH4-β-catenin-AJUBA axis that inhibits cell differentiation and promotes YAP1 activity in inflammation and angiogenesis (***Fig. 5***).

Wnt/β-catenin is a master regulator central to signaling pathways critical for promoting critical biological processes such as cell proliferation, stem cell renewal, cell fate determination, organogenesis, and tissue regeneration[60–65]. Mutations in Wnt/β-catenin are often linked to genetic defects, disease, and cancer[66]. In cancers, Wnt/β-catenin is highly activated, such that it can enhance tumor proliferation, propagate malignant invasiveness and promote the cancer cells' immature, stem-cell-like phenotype. Changes in Wnt/β-catenin have been shown to affect the prognosis of patients with HNSCC[67], and its dysfunction has been shown to promote the development of oral cancer[68]. For these reasons, multiple Wnt/β-catenin modulators have been tested

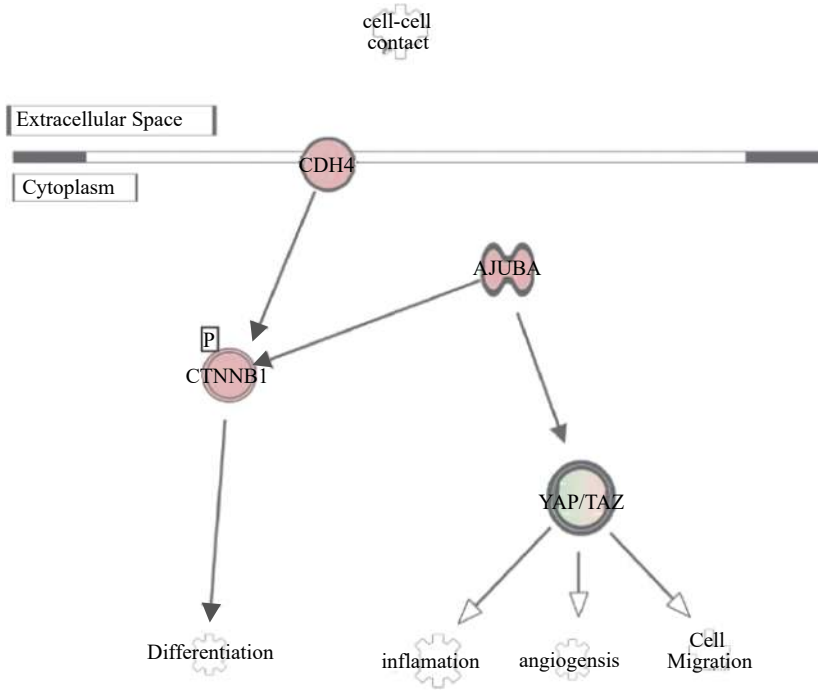

***Fig. 5* Representative diagram of the molecular interactions among AJUBA, CDH4, and CTNNB1.** Cell-cell contacts stimulate CTNNB1 inhibitory effect on osteogenesis differentiation and activate the downstream Hippo pathway effector YAP/TAZ to induce cancer stemness, YAP/TAZ nuclear localization, inflammation, angiogenesis, and cancer cell migration.

in preclinical models for various cancers during the last few years, with some molecules showing some promise *in vivo*[60,65]. However, no drugs of this type have reached clinical trials in head and neck cancers.

Interestingly, despite Wnt/β-catenin's impact on head and neck cancers, there are very few reports of Wnt/β-catenin mutations associated with HNSCC. However, mutations in Wnt/β-catenin upstream regulators such as AJUBA can result in β-catenin stabilization[20,69], which is correlated with de-differentiation and poor prognosis[26,70]. As a scaffolding protein, AJUBA plays an essential role in oncogenesis by regulating major signaling pathways, such as Wnt, JAK/STAT, RAS/ERK, and Hippo. It stabilizes adhesion junctions by linking cadherin and α-catenin to cytoskeletal receptor complexes[71]. AJUBA/SP1 forms an SP1 activating feed-forward loop that functions as a biomarker for pancreatic cancer[18]. Mutations in AJUBA can affect HNSCC sensitivity to treatment with cell cycle inhibitors like AZD7762 and cisplatin[19]. AJUBA functions as an oncogene in esophageal cancer, where it promotes tumor migration[72]. In colorectal cancer, AJUBA promotes EMT and metastasis[22,73−74]. In this study, we investigated how AJUBA, in conjunction with CDH4, cooperates to promote CTNNB1 inhibition of MSC osteogenic differentiation. As well as stimulating Hippo effector proteins YAP/TAZ to inflammation, angiogenesis cancer cell migration, AJUBA seems to be at the crossroads of several critical signaling pathways for cell differentiation and tumor formation. More research is needed to investigate its cancer modulating activity and leverage its properties for therapeutic purposes suitable to cancer stemness, YAP/TAZ nuclear localization, inflammation, angiogenesis, and cancer cell migration.

Acknowledgments

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