IRX5 promotes colorectal cancer metastasis by negatively regulating the core components of the RHOA pathway

Qiangqiang Zhu | Yiqi Wu | Mengli Yang | Zhen Wang | Hailing Zhang | Xinying Jiang | Meng Chen | Tianyu Jin | Ting Wang

1Department of Cell Biology, Nanjing Medical University, Nanjing, Jiangsu, China
2Department of Pathology, The First Affiliated Hospital with Nanjing Medical University, Nanjing, Jiangsu, China
3Department of Molecular, Cellular and Biomedical Sciences, Medical Laboratory Science Program, College of Life Sciences and Agriculture, The University of New Hampshire, Durham, New Hampshire
4Department of Clinic School, Nanjing Medical University, Nanjing, Jiangsu, China

Correspondence
Ting Wang, Department of Cell Biology, Nanjing Medical University, 101 Longmian Avenue, Nanjing, 211166 Jiangsu, China. Email: wangting@njmu.edu.cn

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Abstract
Colorectal cancer (CRC) is one of the most common malignant tumors worldwide. As tumor metastasis is the leading cause of death in patients with CRC, it is important to elucidate the molecular mechanisms that drive CRC metastasis. Studies have shown a close relationship between Iroquois homeobox (IRX) family genes and multiple cancers, while the mechanism by which IRX5 promotes CRC metastasis is unclear. Therefore, we focused on the involvement of IRX5 in CRC metastasis. In this study, analyses of clinical data indicated that the expression of IRX5 was coincided with metastatic colorectal tumors tissues and was negatively correlated with the overall survival of patients with CRC. Functional analysis showed that IRX5 promoted the migration and invasion of CRC cells, accompanied by a large number of cellular protrusions. IRX5-overexpressing cells were more likely to form metastatic tumors in nude mice. Further analysis demonstrated that the core components of the RHOA/ROCK1/LIMK1 pathway were significantly inhibited in IRX5-overexpressing cells. Overexpression of LIMK1 effectively reversed the enhanced cellular motility caused by IRX5 overexpression. Moreover, we found that high levels of IRX5 in intestinal tissues were correlated with the inflammatory response. IRX5 was significantly increased in azoxymethane/dextran sodium sulfate intestinal tissue of mice and IRX5-overexpressing may also enhance chemokines CXCL1 and CXCL8. In summary, our findings suggested that IRX5 promoted CRC metastasis by inhibiting the RHOA-ROCK1-LIMK1 axis, which correlates with a poor prognosis.

KEYWORDS
colorectal cancer, IRX5, metastasis, RHOA pathway

1 | INTRODUCTION

Colorectal cancer (CRC) is one of the most common cancers and a leading cause of cancer-related death worldwide.1,2 Approximately one-quarter of patients with CRC present with metastatic disease; despite the implementation of screening that has effectively reduced CRC mortality in recent years, the 5-year survival rate of patients with metastasis is dismal.3-7 Regardless, little is known about the specific molecular mechanism that drives CRC metastasis.8 The primary goal of this study is to identify novel molecular mechanisms responsible for CRC metastasis.

Overall, the molecular mechanism of tumor cell metastasis is rather complicated. Expression of colorectal neoplasia differentially...
expressed (CRNDE), a long noncoding RNA, was significantly upregulated in a variety of tumors (including CRC) and was associated with tumor metastasis. The Iroquois homeobox 5 (IRX5) gene and CRNDE were in close proximity, and the expression of IRX5 was positively correlated with CRNDE in CRC tissues. IRX family genes, which encoded a class of transcription factors containing a unique IRO box, were highly conserved from invertebrates to vertebrates and play an essential role in development.

There are five recognized types of IRXs: IRX 1, IRX 2, IRX 3, IRX 4, and IRX 5. Over the years, the five different isoforms of IRXs have been shown to be multifunctional proteins affecting tumor growth, invasion, and metastasis. IRX 1 has been reported to inhibit angiogenesis and distant metastasis and was identified as a potential tumor-suppressor gene in head and neck squamous cell carcinoma and gastric cancer, whereas IRX 2 and IRX 3 have been reported to promote tumor progression. The expression level of IRX 2 in osteosarcoma was significantly higher than that in normal tissues, and its high expression was closely related to tumor progression and prognosis. IRX 2 also regulated the expression of matrix metalloprotease-2 and vascular endothelial growth factor (VEGF) in a protein kinase B-dependent manner, thereby promoting the progression of osteosarcoma.

The study clarified that IRX 3 was upregulated in hepatocellular carcinoma cell lines (HepG2 and SMMC7721) and was a direct target of the liver cancer suppressor gene miR-377. These findings suggest that IRX 3 has the potential to promote tumor progression in liver cancer. Studies have shown that VEGF can stimulate the expression of IRX 3 in human microvascular endothelial cells, suggesting that IRX 3 may contribute to tumor angiogenesis in cancer. IRX 4 inhibited cell proliferation, suggesting its tumor-suppressive effect in prostate cancer and oral squamous cell carcinoma.

A previous study showed that IRX 5 promoted G1/S-phase transition in vascular smooth muscle cells via CDK2-dependent activation, and IRX 5 negatively regulated the transforming growth factor β (TGF-β) pathway, thus promoting the transformation of adenoma to cancer. These findings suggest that IRX 5 may play an important role in the early stages of CRC. Inactivation of the TGF-β pathway was considered to be a basic event in the progression of CRC, and the majority of CRC tumors and almost all CRC cell lines harbored such a mutation. However, the role of IRX 5 in later stages (especially in the process of tumor metastasis) of CRC and its related molecular mechanism need to be further studied.

Here, we demonstrate that IRX 5 influences CRC metastasis through the RHOA pathway. Our findings provide new insight into the molecular mechanisms of CRC metastasis and may have implications for the treatment of this cancer.

2 MATERIALS AND METHODS

2.1 Clinical samples

A CRC tissue microarray and associated clinical information were purchased from Shanghai Outdo Biotech Company (HCol-Ade080CD-01; Shanghai, China). An anti-IRX 5 antibody was purchased from Lifespan Biosciences (LS-B3078-50; Seattle, WA), and the working concentration was 5 μg/mL. Immunohistochemical staining (the IRX 5 protein is mainly located in the nucleus and cytoplasm) and the interpretation of the results was performed by a pathologist on a double-blind basis.

2.2 Cell lines and reagents

Human CRC cell lines DLD-1, SW480, LoVo, HT-29, and SW620 were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Stable IRX 5-overexpressing cell lines and matched control cell lines were established using the lentiviral vector system (HANBIO, Shanghai, China). All cell lines were confirmed to be free of mycoplasma contamination and grown in Dulbecco’s modification of Eagle’s medium (Gibco, Grand Island, NY) containing 10% fetal bovine serum (Gibco); an additional 2 μg/mL puromycin was added to the culture medium of the stable cell lines. The selective RHOA/ROCK inhibitor Y-27632 dihydrochloride was purchased from Selleck Chemicals (Houston, TX).

2.3 Western blot analysis

The Western blot analysis procedure has been described in detail. The antibodies used were as follows: anti-IRX 5 (ab56681; Abcam, Cambridge, UK), anti-RHOA (ab187027; Abcam), anti-ROCK 1 (AF0276; Beyotime, Shanghai, China), anti-LIM Kinase 1 (ab108507; Abcam), anti-Vinculin (ab129002; Abcam), anti-rabbit immunoglobulin G (IgG) (7074; Cell Signaling Technology, Beverly, MA), and anti-mouse IgG (7076; Cell Signaling Technology).

2.4 Migration, invasion, and wound-healing assays

For migration analysis, cells were added to Transwell upper chambers (3422; Costar, Cambridge, MA) containing serum-free culture medium. The chambers were incubated in 24-well plates (Costar) containing complete culture medium for a preset time and then removed, and the bottom of the chambers was photographed randomly after fixing, staining, and completely removing the cells in the upper chamber. The number of cells was counted. For the invasion analysis, 20 μg of Matrigel matrix (356234; BD Biosciences, San Jose, CA) was added to each Transwell upper chamber before the above steps were performed. For wound-healing analysis, wounds in cell monolayers in six-well plates (Costar) were produced using a sterile tip. Photographs were taken every 24 hours. For migration and invasion assays, the cells translocated to the bottom of the well were captured by the digital camera, and then all cells were counted by IPP6.0 software. The number of cells in the experimental group was divided by the number of cells in the control group, and the change of relative multiple was obtained. Each statistical result contains three independent repeated experiments, so each statistical chart contains three groups of relative multiple changes and the data of the control group are normalized to “1.”
2.5 | Mouse model

The experiment on animals was approved by the Laboratory Animal Ethics Committee of Nanjing Medical University. Six-week-old nude mice (Beijing Vital River Laboratory Animal Technology Co, Beijing, China) were randomly divided into two groups. Stable IRX5-overexpressing DLD-1 (DLD-1IRX5) cells or control cells (0.5 × 10⁶) were injected into each mouse through the tail vein. After 8 weeks, all mice were euthanized. Pictures of freshly harvested livers were obtained, and the lungs were used for hematoxylin and eosin staining.

BALB/c male mice purchased from the Institute of Model Animals of Nanjing University (Nanjing, China) were used to construct the inflammation-related colon carcinogenesis model. On the first day, mice in the experimental group were intraperitoneally injected with 12.5 mg/kg of azoxymethane (AOM; Sigma Aldrich, St. Louis, Missouri) and the control group were intraperitoneally injected with the same concentration of physiological saline. After 6 days, the mice in the experimental group were continuously fed with 2.5% dextran sodium sulfate (DSS; MP Biomedicals) for 7 days, and then fed with normal drinking water for 14 days, a total of 3 weeks fed is a cycle. The control mice were fed with normal saline. After the third cycle, the mice were euthanized. Intestinal tissue of AOM/DSS mice and control mice were extracted to test the messenger RNA (mRNA) level of IRX5 by real-time polymerase chain reaction (PCR) analysis.

2.6 | Crystal violet staining

Crystal violet staining allows for a clear outline of cells, which helps in precise cell counting and facilitates cell morphology distinction. Fresh cell samples were fixed with 4% paraformaldehyde for 15 minutes and then stained with Crystal Violet Staining Solution (C0121; Beyotime) for 15 minutes and then stained with Crystal Violet Staining Solution (C0121; Beyotime) for 15 minutes and then stained with Crystal Violet Staining Solution (C0121; Beyotime) for 15 minutes and then stained with Crystal Violet Staining Solution (C0121; Beyotime) for 15 minutes and then stained with Crystal Violet Staining Solution (C0121; Beyotime) for 15 minutes and then stained with Crystal Violet Staining Solution (C0121; Beyotime) for 15 minutes and then stained with Crystal Violet Staining Solution (C0121; Beyotime) for 15 minutes.

2.7 | RT² profiler PCR array

Total RNA was isolated from cells using TRIzol Reagent (15596-018; Invitrogen, Carlsbad, CA). Complementary DNA (cDNA) was synthesized using the RT² First-Strand Kit (330401; QIAGEN, Hilden, Germany). Quantitative PCR (qPCR) was performed with Human Cell Motility RT² Profiler PCR Array (PAHS-128ZA; QIAGEN), and the 2−ΔΔCt method was applied to normalize gene expression.

2.8 | Quantitative real-time PCR

Total RNA was isolated from cells using TRIzol Reagent (15596-018; Invitrogen) and cDNA was synthesized using HiScript II Q RT SuperMix (R223-01; Vazyme Biotech, Nanjing, China). qPCR was performed using ChamQ SYBR qPCR Master Mix (Q311-02; Vazyme Biotech) and the following primers: IRX5: Forward, 5′-TCCTATCCCGAGGGCTACTT-3′ and Reverse, 5′-GATGGCATGGACTGGTGTCA-3′. The 2−ΔΔCt method was used to normalize gene expression.

2.9 | Enzyme-linked immunosorbent assay

Exocrine proteins CXCL1 and CXCL8 were detected using a human growth-regulated oncogene α enzyme-linked immunosorbent assay (ELISA) kit (CSB-E09150h; CUSABIO, Wuhan, China) and human interleukin 8 ELISA KIT (CSB-E04641h; CUSABIO), respectively, according to the protocol provided by the manufacturer.

2.10 | Transient transfection

A LIMK1 plasmid (2.5 μg) or the control vector was transfected into cells at 80% confluence using Lipofectamine 3000 (Invitrogen) following the manufacturer’s protocol.

2.11 | Public databases

The public databases used in this study were as follows: Gene Expression Profiling Interactive Analysis (GEPIA) (http://geopia.cancer-pku.cn/; Accessed 10 January 2018); Catalog Of Somatic Mutations In Cancer (COSMIC) (http://cancer.sanger.ac.uk/cosmic; Accessed 18 January 2018); Genomatix (http://www.genomatix.de/; Accessed 18 January 2018); and the database for annotation, visualization and integrated discovery (DAVID) (http://www.genomatix.de/; Accessed 18 January 2018).

Gene Expression Omnibus (GEO) on-line network analysis tool "GEO2R" was used to analyze the differential expression of "IRX5" of the AOM/DSS-induced colitis-associated CRC mouse model by the dataset “GSE31106.” The specific analysis method was carried out according to the steps indicated by the website (https://www.ncbi.nlm.nih.gov/geo/info/geo2r.html).

2.12 | Statistical analysis

GraphPad Prism 5.0 (GraphPad Software, Inc, San Diego, CA) was employed for statistical analysis of the experimental data, and the results are presented as the mean ± SD. P < .05 between paired groups were considered significant.

3 | RESULTS

3.1 | IRX5 was highly expressed in CRC tissues and associated with tumor metastasis

We used immunohistochemistry to detect IRX5 protein levels in clinical samples. The clinic features of patients are shown in Table S1. Samples were divided into three categories according to the proportion of positively staining cells: low staining for which the proportion of positive cells was less than 10%, 0 points; moderate staining for which the proportion of positive cells was 10% to 50%, 1 point; strong staining for which the proportion of positive cells was more than 50%, 2 points. On the basis of IRX5 immunohistochemical
staining results, normal tissues \((n = 27)\) mainly displayed low and moderate staining, whereas adenomas \((n = 11)\), primary tumors \((n = 16)\), and metastatic tumors \((n = 9)\) exhibited strong staining (Figure 1A and 1B). There was significantly higher staining in the primary paracancerous tissues of stage IV compared with stages I/II/III tumors (Figure S1). Because stage IV tumors had distant metastases, the levels of IRX5 in paracancerous tissues may reflect the increased propensity for cancer metastasis.

The above staining standards can only reflect the difference in the number of positively stained cells in the sample and not the difference in staining intensity. Although both primary and metastatic tumors stained strongly (more than 50% of the positive cells), the level of IRX5 staining intensity of metastatic tumors was significantly higher than that of primary tumors (Figure 1C). This reflected the correlation between high expression of IRX5 and CRC metastasis. Tumor metastasis will have a significant impact on the 5-year survival of patients with CRC, and because the level of IRX5 expression is associated with tumor metastasis, it is likely to be associated with patient survival. Thus, we utilized the public database GEPIA (http://gepia.cancer-pku.cn/) to analyze the relationship between IRX5 expression and survival among patients with CRC. The clinical sample data in this database originated from The Cancer Genome Atlas and Genotype-Tissue Expression databases. Compared with relatively low expression, relatively high expression of IRX5 was associated with a significantly increased risk of death (hazard ratio = 1.9) and short median survival time (Figure 1D).

### 3.2 IRX5 enhanced the mobility of CRC cells

The occurrence of cancer metastasis is closely related to changes in the motility of tumor cells. Therefore, we evaluated the effect of IRX5 on the motility of CRC cells. To this end, we used the CRC cell lines DLD-1 and SW480 (IRX5 expression levels in these cell lines are very weak) to construct two cell lines DLD-1\(^{IRX5}\) and stable IRX5-overexpressing SW480\(^{IRX5}\) stably overexpressing IRX5 and the control cells (DLD-1 stably transfected with control vector [DLD-1\(^{Control}\)] and SW480 stably transfected with control vector) (Figure 2A and 2B). DLD-1\(^{IRX5}\) cells exhibited much greater migration.

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**Figure 1** IRX5 is highly expressed in CRC tissues and is associated with tumor metastasis. A, Typical pictures and corresponding high magnification of IRX5 immunohistochemical staining on CRC tissue microarray slides. B, Statistical analyses of IRX5 immunohistochemical staining differences between adenomas, primary tumors, metastatic tumors, and normal tissues (**\(P < .001\)**). C, Comparison of IRX5 staining intensities of primary and metastatic tumors. D, Overall survival analysis based on IRX5 expression in patients with CRC. CRC, colorectal cancer; HR, hazard ratio; IHC, immunohistochemistry; IRX, Iroquois homeobox; TPM, transcripts per million [Color figure can be viewed at wileyonlinelibrary.com]
FIGURE 2  Continued.
Previous reports have shown that the RHOA pathway inactivation promotes the migration and invasion of CRC cells. In our study, we verified this in three CRC cell lines (DLD-1, SW480, and LoVo) using Y27632, a specific inhibitor of the RHOA/ROCK1 pathway. Y27632 treatment significantly enhanced the migration of these three CRC cell lines (Figure 3C).

The above results demonstrate that IRX5 affects cell motility by inhibiting the RHOA-ROCK1-LIMK1 pathway. However, whether this pathway plays a key role requires further investigation. Therefore, we overexpressed LIMK1 in DLD-1IRX5 and SW480IRX5 cells (Figure S3) and found that upregulation of LIMK1 effectively reversed the enhanced migration caused by IRX5 (Figure 3D and E). These results confirm that the RHOA-ROCK1-LIMK1 pathway has a vital role in IRX5-mediated cell migration.

3.3 | IRX5 increased cell motility by reducing RHOA-ROCK1-LIMK1 pathway activity

The above results suggest that IRX5 is directly related to cell motility. We, therefore, applied Human Cell Motility RT2 Profiler PCR Array to detect target genes downstream of IRX5. A total of 36 genes exhibited significant differences in expression between DLD-1IRX5 and DLD-1Control cells (Table 1), and nearly one-third of these genes (shown in bold) are involved in the RHOA signaling pathway. It is worth noting that the three key genes (RHOA, ROCK1, and LIMK1) of the RHOA pathway were all downregulated, suggesting that the RHOA pathway is likely an important pathway downstream of IRX5.

In addition, we verified the PCR array results for DLD-1IRX5 cells at the protein level (Figure 3A). Moreover, the key components of this pathway were also downregulated in SW480IRX5 cells (Figure 3B). One of the most direct functions of the RHOA pathway is the reorganization of the actin cytoskeleton, and phalloidin staining demonstrated that IRX5 upregulation induced a significant change in the arrangement of F-actin (Figure S2). In DLD-1Control cells, F-actin was mainly enriched in the vicinity of the cell membrane; in DLD-1IRX5 cells, F-actin was mostly found in the cytoplasm, and a large number of fiber bundles protruded from the cell membrane. This staining pattern was consistent with the observed changes in cell morphology (Figure 2G). These results indicate that IRX5 affects the RHOA pathway and can cause corresponding biological effects.

3.4 | Upregulation of IRX5 was related to inflammation

The above results reveal the role of IRX5 in CRC progression and metastasis. Next, we examined the mechanisms that likely drive the overexpression of IRX5 in CRC.

We used the GEPIA database to detect the level of IRX5 mRNA in CRC tumor and normal tissues and found significantly higher IRX5 mRNA levels in tumor tissues (Figure 4A), suggesting transcriptional activation of the IRX5 gene. These results indicate that elevated IRX5 protein levels in cancer tissues are due to upregulation of the IRX5 gene. Events that affect gene expression include point mutations, gene copy number changes, promoter methylation, and transcription factor regulation. However, COSMIC (http://cancer.sanger.ac.uk/cosmic) database analysis revealed very few cases of point mutation, copy number change, or promoter methylation in CRC tissues (Figure 4B). In addition, treatment of five commonly used CRC cell lines with the demethylation agent 5-aza-2′-deoxycytidine did not effectively alter IRX5 mRNA levels (Figure 4C), indicating no obvious methylation events in the cultured CRC cell lines. Therefore, we sought to determine that IRX5 upregulation is due to upstream transcriptional regulators.

We used the Genomatix website (http://www.genomatix.de/) to predict possible transcription factors binding to the IRX5 promoter region (2000 bases upstream of the 3′-untranslated region) and then performed the pathway enrichment analysis on these predicted transcription factors on the DAVID website (https://david.ncifcrf.gov/home.jsp). The results showed enrichment of these transcription factors, indicating that IRX5 is regulated by various transcription factors, including c-Myc, E2F1, and NFκB.
TABLE 1 Differentially expressed genes in DLD-1IRX5 cells compared with DLD-1control cells

| Gene symbol | Fold regulation | Gene symbol | Fold regulation | Gene symbol | Fold regulation |
|-------------|----------------|-------------|----------------|-------------|----------------|
| ACTN1       | -2.0333        | FGF2        | -2.0276        | PTK28       | -2.0038        |
| AKT1        | -2.0108        | IGF1        | 3.9962         | RASA1       | -2.0303        |
| ARHGDI A    | -1.9753        | ITGB2       | 4.0412         | RHOA        | -2.023         |
| ARHGEF7     | -1.9877        | ITGB3       | 1.9969         | RHOB        | -2.0163        |
| BAIAP2      | -2.0167        | LIMK1       | -2.0195        | ROCK1       | -2.0059        |
| BCR1        | -2.0015        | MET         | -2.0162        | STAT3       | 1.9844         |
| CAPN1       | -2.026         | MSN         | 8.5505         | SVIL        | -2.0099        |
| CRK         | -2.0193        | MYH10       | -1.9909        | TGFβ1       | -1.9935        |
| DPP4        | -1.9761        | MYL9        | -1.9827        | VCL         | -2.0065        |
| EGF         | 1.9574         | PDL1        | -2.0076        | VEGFA       | 1.9998         |
| ENAH        | -3.9628        | PRKCA       | -1.992         | VIM         | 7.9969         |
| FAP         | -2.1374        | PTEN        | -2.0223        | WASL        | -1.9961        |

Note: The 2−ΔΔCt method was used to normalize gene expression. GAPDH served as the reference gene.

Abbreviations: DLD-1Control, DLD-1 stably transfected with control vector; DLD-1IRX5, stable IRX5-overexpressing DLD-1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

...factors in cancer and immune system-related diseases (Table 2). This finding indicated that tissues and cells under these environmental conditions most likely have high levels of IRX5.

The immune system promotes the development of tumors by producing an inflammatory environment, and most solid tumors have immune cell infiltration. As a prior study showed that tumor necrosis factor α strongly induces expression of IRX family genes in keratinocyte, we speculated that inflammation may be the driving factor of IRX5 overexpression in CRC.

To test the relationship between IRX5 and inflammation, we built the AOM/DSS mice model which has been verified as a successful inflammation-related colon carcinogenesis model. Intestinal tissue of AOM/DSS mice showed a significant upregulation of IRX5 compared with that of the control group, suggesting IRX5 may play a role in the progression of inflammatory cancer (Figure 4D). Moreover, the data from the GEO database showed that levels of IRX5 were robustly elevated with abnormal tissue progression during the development of colitis-associated CRC in mice induced by AOM/DSS (Figure 4E). These results demonstrate that upregulation of IRX5 in CRC tumors is closely related to the inflammatory response.

CXCL1 and CXCL8 have a strong chemotactic effect on immune cells, and expression of these two chemokines was significantly increased in IRX5-overexpressing cells (Figure 4F and 4G). Therefore, it is likely that IRX5 expression and the inflammatory response are mutually reinforcing.

4 DISCUSSION

In this study, we for the first time explored the role of IRX5 in CRC metastasis and showed that IRX5 affected cell motility via the RHOA-ROCK1-LIMK1 pathway. Given the relationship between IRX5 and overall survival in patients with CRC, we believe that IRX5 plays an important role in the later stages of the disease. Taking into consideration the previous studies have shown that IRX5 participates in cell cycle and tumorigenesis. It appears that IRX5 is involved in the entire process of CRC.

Transgene and gene knockout techniques are the basic means for examining gene function. In this study, we only used transgenic technology to overexpress IRX5 in CRC cells without using gene knockout to downregulate IRX5 levels. One of the reasons for this was that the overexpression of IRX5 in cells may need to be driven by an inflammatory microenvironment and that the expression of IRX5 immediately returns to a very low level under noninflammatory conditions, without effective silencing. This also occurs in isolated cultured CRC cell lines. Indeed, we surveyed the IRX5 expression in a panel of CRC cell lines (Figure S4) and found little difference in the level of IRX5 among them. We detected the IRX5 protein expression in DLD-1 and SW480 cells (Figure 2A and 2B), and the results indicated very weak expression in these cell lines. Because we cannot replicate the complex inflammatory microenvironment in vivo and the key inflammatory factors driving expression of IRX5 have not yet been determined, we did not use the gene knockout technique to further explore the function of IRX5. Furthermore, mammalian IRX genes function redundantly during embryonic development, and loss of function of individual IRX genes can be compensated for by expression of other family members. For example, IRX3 and IRX5 function redundantly in mouse heart development. Considering that IRX3 and IRX5 are coexpressed in most human tissues, they might also function redundantly in cancer progression. Thus, knocking out IRX5 alone in cells does not cause significant functional changes, and double knockdown will inevitably affect the analysis of single-gene function. Therefore, it remains under debate whether the gene knockout technique is applicable to the study of single IRX gene function.

It has been reported that IRX5 was significantly upregulated in tongue squamous cell carcinoma (TSCC) tissues and cell lines.
FIGURE 3  IRX5 increases cell motility by reducing RHO-ROCK1-LIMK1 pathway activity. A, Western blot analyses of RHOA, ROCK1, and LIMK1 in DLD-1IRX5 cells. All statistical results were obtained from three independent replicates (*P < .05; **P < .001). B, Western blot analyses of RHOA, ROCK1, and LIMK1 in SW480IRX5 cells. All statistical results were obtained from three independent replicates (*P < .05; **P < .001). C, Migration results for three CRC cell lines. All statistical results were obtained from three independent replicates (**P < .01; ***P < .001). The concentration of Y27632: 10 μM. DLD-1, SW480, or LoVo cells (2 × 10^5) were adhered to Transwell upper chambers and then incubated for 12 hours. Migration results of (D) DLD-1IRX5 and (E) SW480IRX5 cells. All statistical results were obtained from three independent replicates (**P < .01). Three experimental groups of both stable IRX5-overexpressing cell lines were established: A (the empty vector-transfected into control cells), B (the empty vector-transfected into IRX5-overexpressing cells), and C (the LIMK1-overexpression plasmid were transfected into IRX5-overexpressing cells). Stable IRX5-overexpressing or control cells (8 × 10^4 for DLD-1IRX5 and DLD-1Control cells; 10 × 10^4 for SW480IRX5 and SW480Control cells) were adhered to transwell upper chambers and then incubated for 24 hours. DLD-1Control, DLD-1 stably transfected with control vector; DLD-1IRX5, stable IRX5-overexpressing DLD-1; DMSO, dimethyl sulfoxide; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IRX, Iroquois homeobox; SW480Control, sW480 stably transfected with control vector; SW480IRX5, stable IRX5-overexpressing SW480 [Color figure can be viewed at wileyonlinelibrary.com]
FIGURE 4  Continued.
Overexpression of IRX5 was associated with TSCC cell migration and invasion, which coincided with our data to some extent. Moreover, IRX5 exerted this role through targeting osteopontin promoter and the nuclear factor κB pathway also participated in promoting this progress.42

In addition, our data support the notion that inactivation of the RHOA pathway plays a key role in IRX5-mediated CRC metastasis. However, as many studies have shown that RHOA was overexpressed in tumor tissues compared with normal tissues, the function of the RHOA pathway in CRC is controversial.43,44 We, therefore, performed a correlation analysis of IRX5 with RHOA, ROCK1, and LIMK1 in the above-mentioned AOM/DSS-induced colitis-associated CRC mouse model, and the results showed the expression levels of RHOA, ROCK1, and LIMK1 to all be sharply increased at the very beginning of drug treatment followed by a downward trend (Figure S5). This suggests that activation of the RHOA pathway in cancer tissues is likely to be a stress response caused by certain factors, such as inflammation and drugs. Because cancer patients may undergo treatments, such as radiotherapy and chemotherapy before tumor resection, these genes are inevitably stimulated by certain therapeutic factors in clinical samples and analysis, thus cannot accurately reveal their true levels. Accordingly, the function of the RHOA pathway should be evaluated from the perspective of the entire tumorigenesis process rather than through a simple comparison of expression differences between tumor and normal tissues. In contrast, the IRX5 expression exhibited an upward trend in this process. With regard to the sharp decline in the 20th week, in addition to experimental error in the sample itself, the most likely reason is that at this time, the intestinal lesions had been transformed into adenomas and these dense adenomas prevented most cells from fully contacting the inflammatory environment; therefore, this driver of IRX5 expression was absent.

Overall, the occurrence of tumor metastasis does not depend solely on the enhancement of cell motility, and high expression of IRX5 can also confer a number of other advantages on tumor cells. For instance, the levels of certain growth factors (eg, endothelial growth factor and VEGFA) were high in IRX5-overexpressing cells (Table 1), which may promote the formation of tumor-infiltrating blood vessels. All of the functions of IRX5 contribute to distant metastasis.

In this study, we found a correlation between IRX5 overexpression and the inflammatory response. On the one hand, the immune system can drive IRX5 expression by creating an inflammatory environment, thereby conferring many oncogenic properties to cells; on the other hand, the immune system has an inherent ability to eliminate cancerous cells. The final result we observed was that most tumor cells showed strong expression of IRX5, which appears to be the result of immune system screening. The cancer immunoediting theory suggests that the immune system overlooks some tumor cells, which therefore escape the fate of being cleared from the body.32 This indicates that IRX5 may be involved in the mechanism of immune escape.

Inactivation of the TGF-β pathway is considered to be the third basic event in the development of CRC, in addition to APC and TP53 mutation inactivation.6 We found that IRX5 decreased the expression of TGFβ1 (Table 1). Moreover, it has been reported that IRX5

| KEGG pathway term                  | P value     |
|------------------------------------|------------|
| Hepatitis B                        | 5.20E-10   |
| Transcriptional misregulation in cancer | 3.40E-09  |
| HTLV-I infection                   | 5.40E-09   |
| Maturity onset diabetes of the young | 3.50E-05  |
| Prostate cancer                    | 3.70E-05   |
| Acute myeloid leukemia             | 5.30E-05   |
| Signaling pathways regulating pluripotency of stem cells | 6.60E-05 |
| Pancreatic cancer                  | 1.10E-04   |
| Pathways in cancer                 | 1.30E-04   |
| Viral carcinogenesis               | 2.90E-04   |
| Inflammatory bowel disease (IBD)   | 1.70E-03   |
| Chronic myeloid leukemia           | 2.00E-03   |
| Cell cycle                         | 1.30E-02   |
| Measles                            | 1.90E-02   |
| Thyroid cancer                     | 2.20E-02   |
| Hippo signaling pathway            | 2.50E-02   |
| Small cell lung cancer             | 2.70E-02   |
| Bladder cancer                     | 3.50E-02   |
| MicroRNAs in cancer                | 3.50E-02   |

Note: These results are derived from the DAVID website. P < .05 were considered significant.

Abbreviations: DAVID, The database for annotation, visualization and integrated discovery; IRX, Iroquois homeobox; HTLV, human T-cell leukemia virus type; AOM, azoxymethane; COAD, Colon adenocarcinoma; MHC, Major Histocompatibility Complex; APC, adenomatous polyposis coli; TP53, tumor protein p53; TGF, transforming growth factor; CRC, colorectal cancer; DLD, human normal colorectal adenocarcinoma cell line; ROCK1, Rho kinase 1; LIMK1, LIM-kinase 1; ROCK2, Rho kinase 2; LIMK2, LIM-kinase 2; DSS, dextran sodium sulfate; GEO, Gene Expression Omnibus; IRX, Iroquois homeobox; mRNA, messenger RNA; PCR, polymerase chain reaction; SW480IRX, stable IRX5-overexpressing SW480 [Color figure can be viewed at wileyonlinelibrary.com]
downregulated the expression of SMADs and core components of the TGF-β pathway. Thus, IRX5 may confer growth advantages to tumor cells by inhibiting the TGF-β pathway, thereby promoting the transformation of adenoma to cancer.

On the basis of our findings, we propose a model for describing the possible role of IRX5 in the progression and metastasis of CRC (Figure 5). In the precancerous stage, excessive proliferation of intestinal epithelial cells initiate the inflammatory response, and inflammatory factors stimulate a portion of intestinal epithelial cells with high IRX5 expression. These cells successfully avoid immune surveillance and gradually form an early adenoma. Increased levels of inflammatory factors enhance the inhibitory effect of IRX5 on the TGF-β pathway, accelerating the transformation from a precancerous lesion to cancer. After the disease has progressed into a malignant tumor, a large number of immune cells infiltrate into the cancer tissue, further increasing the level of IRX5. Cell motility is enhanced by the inhibition of the RHOA pathway. The combined action of these multiple factors eventually leads to CRC metastasis. CRC, colorectal cancer; IRX, Iroquois homeobox; TGF-β, transforming growth factor β [Color figure can be viewed at wileyonlinelibrary.com]

FIGURE 5 Proposed model relating the function of IRX5 in the progression and metastasis of CRC. The inflammatory response results in elevated expression of IRX5. Excessive proliferation of intestinal epithelial cells confers immune surveillance escape and results in early adenoma formation. IRX5 confers malignant cell growth advantage by negatively regulating the TGF-β pathway, thereby enabling adenomas to progress to cancer. A large number of immune cells infiltrate into the cancer tissue to further increase the level of IRX5. Cell motility is enhanced by the inhibition of the RHOA pathway. The combined action of these multiple factors eventually leads to CRC metastasis. CRC, colorectal cancer; IRX, Iroquois homeobox; TGF-β, transforming growth factor β [Color figure can be viewed at wileyonlinelibrary.com]

CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

ORCID

Ting Wang http://orcid.org/0000-0002-4245-4684

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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