Present status and expectation of aristaless-related homeobox (ARX) in endocrine pancreas

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ABSTRACT The aristaless-related homeobox (ARX) gene has become one of most frequently mutated genes which is closely linked with development of the vertebrate central nervous system; however, the molecular and clinical bases of its function in the proliferation and differentiation of the endocrine pancreas have not, to date, been systematically characterized. ARX is considered as a regulator which determines endocrine cell fate and a bio-marker of the pancreatic α-cell. Disruption and mutation of ARX are found to lead to the deletion and reduction of α-cells both in mice models and in humans. Furthermore, expression of ARX is regulated by multiple transcription factors involved in development of the pancreas, such as Ngn3, Isl1, Nkx2.2 and Nkx6.1. Taken together, given the vital importance of glucagon in diabetes treatment, it is possible that ARX may down-regulate exorbitant glucagon levels by reducing the number of α-cells as a direct target; thus, the role of ARX in the maintenance of α-cell identity and quantity should be investigated and summarized. This article mainly focuses on the role of ARX in the endocrine pancreas, introduces the ARX-related animal model and transcription factors, and highlights the latest advances in our understanding in order to provide a clearer theoretical foundation for future scientific research.

KEY WORDS: ARX, endocrine pancreas, transcription factor, mouse model, apoptosis

Introduction

ARX, the Aritaless-related homeobox gene, was isolated and identified from mouse cDNA library by Hirohito Miura et al., in 1997 (Miura et al., 1997). Initially, ARX was characterized in embryos of zebrafish and mice, and found to possess remarkable similarity with Drosophila gene aristaless (Miura et al., 1997). The patients with X-linked diseases such as West syndrome, infantile spasms syndrome, lissencephaly with ambiguous genitalia and intellectual disability, non-syndromic mental retardation and Partington syndrome, were identified to experience ARX gene mutations (Gecz et al., 2006, Shoubridge et al., 2010). Thus the human ortholog was discovered in 2002 followed by above mentioned observations (Bienvenu et al., 2002, Kitamura et al., 2002, Stromme et al., 2002a, Stromme et al., 2002b).

To date, many cases about different ARX mutations carried in human families and several studies on the function and mechanism of ARX has been reported (Friocourt and Parnavelas, 2010, Friocourt and Parnavelas, 2011, Friocourt et al., 2006, Gecz et al., 2006, Olivetti and Noebels, 2012, Shoubridge et al., 2010). However, expression and role of ARX is not only defined in brain. In fact, the disruption or other mutations of ARX are found to lead the deletion or reduction of pancreatic α-cells both in mice models and human being (Colombat et al., 2003, Itoh et al., 2010, Wilcox et al., 2013b, Xu et al., 2013). During this process, Glucagon secreted from α-cell is also deficiency. These evidences indicate that ARX not only play pivotal role in the development and proliferation of endocrine cells, but also have the potential effect on glucose homeostasis and glycemic control. All of these illustrate the importance of ARX in development and differentiation of multiple endocrine cells.

Since the discovery of ARX, several reviews have introduced its function in vertebrate central nervous system (Friocourt and Parnavelas, 2010, Friocourt and Parnavelas, 2011, Friocourt et al., 2006, Olivetti and Noebels, 2012). It also makes progress in...

Abbreviations used in this paper: ARX, aristaless-related homeobox.

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endocrine research and is necessary to review, analyze and evaluate achievements already obtained in the past 13 years. In this article, the currently available published ARX mutants in pancreas with the associated clinical and experimental phenotypes are listed and summarized. The recent molecular findings and underlying mechanisms are compared and discussed in order to provide a clearer look into the inheritance and pathogenesis of ARX.

**ARX gene and protein**

ARX gene is located at the genomic region Xp22 with a span of 12.25kb which holds five coding exons giving rise to a 1686bp ORF (open reading frame) in human (Gecz et al., 2006, Shoubridge et al., 2010). The mRNA produced by ARX is 2.8-kb, and the protein which encoded by ARX gene is made up of 562 amino acid, forming four characteristic polyalanine tracts where most of the mutations occur (Gecz et al., 2006, Shoubridge et al., 2010, Yu et al., 2014).

The Arx protein regulates gene expression as a nuclear transcriptional factor in a variety of tissues and organs. Until now, Arx has been found expression predominately in the testes (Kitamura et al., 2002, Miyabayashi et al., 2013), skeletal muscle (Biressi et al., 2008[18], pancreatic endocrine cell (Collombat et al., 2003), enterendocrine cell (Beucher et al., 2012, Du et al., 2012, Terry et al., 2015), fetal and adult brain (Ohira et al., 2002, Yoshihara et al., 2005).

In mouse embryo, Arx is expressed starting at embryonic day 9.5 (E9.5) and thereafter throughout whole development stages (Collombat et al., 2003, Miura et al., 1997). It is supposed to be expressed first in the endocrine progenitor cells and then restricted to α-cell (Collombat et al., 2003).

**Mice models**

To understand its influence in development and differentiation of pancreas, several mice models were made for the investigation. Thus, in this paper, we summarized the physiologic and metabolic feature among these established models. The results may help us understand how ARX gene functions in pancreas of mammalians.

**ARX-null mice model**

Collombat et al., first established ARX-null mice by homologous recombination in embryonic stem (ES) cells. The β-galactosidase gene and the neomycin resistance gene were used to replace octapeptide, α-helix 1 and about half α-helix 2 of the homeodomain. Retarded growth and dehydration was observed on ARX-null mice. Hyperglycemia was appeared 2 days after birth followed by death (Collombat et al., 2003).

**ARX gain-of-function mice model**

Gain-of-function mice were also generated by Collombat et al., in 2007 using transgenic method which showed growth retardation and pancreatic hypoplasia with a shortened life span of 2-12 weeks. Before death, the blood and urinary sugar level was dramatically elevated (Collombat et al., 2007).

**Deficient for ARX in pancreatic endocrine cell mice model**

Aidan S. Hancock et al., established ARX-deficient mice by solely deleted ARX gene in the pancreatic endocrine cells. Comparing with the wild-type, ARX-deficient mice exhibited no difference in body weight, blood glucose. The mice were healthy and the glucose tolerance test was improved and basal hepatic glucose level was reduced and the quantities of glcogenin in the liver were increased (Hancock et al., 2010).

**Deficient for ARX in mature α-cell mice model**

Two kinds of conditional knockout mice models in which the ARX...
gene had been ablated specifically in α-cell were set by Catherine Lee May (Wilcox et al., 2013b) and Patrick Collombat’s laboratories. One of them established by Patrick Collombat’s laboratory could selective inhibited Arx gene in α-cell at any developmental stages (Courtney et al., 2013).

Both of the two mice models were found viable and fertile, their life expectancy and basal glycemia remaining within normal range. In addition, they showed an increased capacity to counteract the glucose bolus with a lower peak in glycemia in intraperitoneal glucose tolerance test (Courtney et al., 2013, Wilcox et al., 2013b).

Other ARX mutant mice models
Kunio Kitamura et al., introduced three ARX mutant mice in 2009 (Kitamura et al., 2009). Among these mice models, two types were used in the endocrinology research.

GCG7 mutant mice
At residue 330 of the mouse ARX gene, seven GCG-triplets were inserted to generate GCG7 mutant (Kitamura et al., 2009). Most of them survived for 3-4 months, few last till 5-6 months (Kitamura et al., 2009, Xu et al., 2013). GCG7 mutant mouse was analyzed as ARX expanded model in genetic study, and its ARX mRNA level was significantly down-regulated to approximately 30% of wild-type level in E15.5 pancreata (Wilcox et al., 2013a).

P355L mutant mouse
The proline residue at the position of 355 was changed to leucine to generate P355L mutant mice. These mice survived for more than 6 months and western blotting showed that the Arx protein from forebrain was also deceased but not as severe as GCG7 mutant mice and the total mRNA level from embryo was also not changed (Kitamura et al., 2009).

ARX-related transcription factors and regulators
After realized the phenotypes shown in these mice models mentioned above, function of ARX in islet of Langerhans raised researchers’ attention. In the network of development and survival of endocrine cells, the role of ARX and the transcription factors related to it should be fully studied. Here we introduce several transcription factors and describe the relationship with ARX via researches since 2003, which is shown in Fig. 2, aim to comprehensive understand the molecular mechanism of ARX in pancreas and expand the way of thinking for intensive research in future.

Ngn3
The bHLH transcription factor neurogenin3 (Ngn3) is an early marker of cells differentiating toward a primary endocrine fate (Habenner et al., 2005, Rukstalis and Habener, 2009). Ngn3-null mice exhibit endocrine precursor cell generation failure while over-expression results in acceleration of differentiation (Apelqvist et al., 1999, Gradwohl et al., 2000, Gu et al., 2002, Johansson et al., 2007) and the Arx protein is not expressed in pancreas (Collombat et al., 2003), which suggests a potential role in ARX downstream of NGN3 in islet of Langerhans developmental processes.

Pax4
The transcription factor Pax4 is a paired-box homeoprotein functions early in the development of islet cells to promote the differentiation of β- and δ-cells (Habenner et al., 2005, Napolitano et al., 2015, Sosa-Pineda et al., 1997). Arx and Pax4 is a pair of reciprocal repression transcription factors. Pax4 promotes β- and δ-cell fates, whereas Arx favors α-cell destiny (Collombat et al., 2003). Both of them act as transcriptional repressors that control the expression level of another one to mediate the proper endocrine fate allocation. It is noteworthy to mention that, Arx has maintained its role in α-cell differentiation from fish to mammals, but Pax4 has no apparent function in the formation of β-cell in zebrafish embryos which indicates that Pax4 acquired its essential role in β-cells differentiation quite late in vertebrates’ evolution (Djotsa et al., 2012).

Isl1 and Ldb1
The LIM homeodomain protein islet1 (Isl1) could be detected in multiple tissues and represents the first known activator of ARX transcription in α-cells (Zhuang et al., 2013). Experiment results indicate ISL1 gene is required for the development of dorsal pancreatic mesenchyme and essential for the formation and proliferation of endocrine cells (Ahlgren et al., 1997, Guo et al., 2011). The LIM domain-binding protein 1 (Ldb1) is essential for Isl1 biological activity as a cofactor (Agulnick et al., 1996, Makarev and Gorivodsky, 2014) which distributed in the early pancreatic epithelium and surrounding mesenchyme, and finally expressed in mature endocrine and ductal cells. Removal of Ldb1 in embryonic endocrine cells leads to the down-regulation of ARX expression (Hunter et al., 2013).

Tie1, Tie2 and Tie3
The Groucho family members, containing transducin-like enhancer of split1 (Tie1), Tie2, Tie3 and Tie4 act as transcriptional co-repressors and are overlappingly expressed during the development of pancreas (Chen and Courey, 2000, Jennings and Ish-Horowicz, 2008). Tie2 can interact with several transcription factors involved in development and proliferation of pancreas to modulate the repressive abilities of ARX in β-cell line (Hoffman et al., 2008). Ectopic expression of Tie3 in α-cells represses glucagon and ARX. And the function of Tie1 is similar to Tie3 in endocrine cells (Metzger et al., 2014).
**Nkx2.2 and Nkx6.1**

The member of NK2 family transcription factor, Nkx2.2 is required for the development and differentiation of pancreatic endocrine cells (Balderes et al., 2013, Sussel et al., 1998). Nkx2.2 regulates expression of ARX as a transcription repressor in endocrine cells. Deficiency of Nkx2.2 leads to a severely loss of β-cells and reduction of α- and γ-cells, and increase of ε-cells in mouse embryo (Kordowich et al., 2011, Mastracci et al., 2011). Nkx6.1 lies downstream of Nkx2.2 in the development of islet (Sander et al., 2000). The expression of Nkx6.1 persists in multipotent pancreatic progenitor in early developmental stage. Nkx6.1 and Isl1 regulate ARX antagonistically for determining α- and β-cell fate, in which Nkx6.1 binds and represses ARX via occupies the conserved Re control domain (Schaffer et al., 2013).

**Activin A and B**

Activins, including activin A, activin AB and activin B are disulfide-linked homodimers of inhibin β subunits (Dani, 2013, Refaat, 2014), which suppress critical α-cell gene expression, including ARX, in α-cells and enhances the expression of β-cell genes, including PAX4, in β-cells (Andrzejewski et al., 2015, Mamin and Philippe, 2007).

**Pem16**

PR domain-containing 16 (Pem16) is expressed in fetal pancreatic epithelial cells including Ngn3⁺ cells, and deficiency of Pem16 leads to significantly increase of ARX expression in endocrine cells, together with hyperplasia of α-cells and γ-cells (Sugiyama et al., 2013).

**Rb and E2f1**

The retinoblastoma protein (Rb) is always mentioned with the transcription factor E2f1 as a whole required for cell cycle progression in autophagy or apoptosis pathway (Laine and Westermarck, 2014, Udayakumar et al., 2010, Wu and Yu, 2009). Rb phosphorylation leads to its dissociation from E2f1, and the inhibition of transactivation is removed (Mayank et al., 2014, Sahin and Sladek, 2010, Sun et al., 2010). It is found that a conserved E2f1 binding site locates in exon 2 of ARX gene, and Rb blocks the ARX gene repression by binding to E2f1 (Cai et al., 2013). Overall, the absence of Rb leads to an increase in E2f1 and repression of Arx, in contrast, E2f1 knockdown restored Arx levels in α-cells.

By the above experimental results, location of Arx is clearly distinguished in the signal network for development and specification of pancreatic endocrine cells. Some multipotent pancreatic progenitors express the endocrine specific transcription factor Ngn3 and differentiate into committed islet endocrine precursors, and then Arx is expressed in the process from endocrine precursors to α-cells. Though analysis of the role and starting expression time of other transcription factors which repress expression of Arx, there are reasons to believe that Arx is inhibited by Nkx2.2, Nkx6.1 and other β-cell specific transcription factors mentioned above in the development of β-cell.

**The role of ARX in the pancreas**

**Differentiation and conversion**

Deficiency of Arx leads to the absence of α-cell and decrease in partial glucagon and ghrelin co-expressing cell number which does not give rise to...
the total endocrine cell mass. That is to say, variation of α-cell number is accompanied by the opposite changes in β- and δ-cell number (Collombat et al., 2003, Hancock et al., 2010). It is general thought that islet subtype destiny is directed by cross-repression of the reciprocal transcription factors Arx and Pax4, and the simultaneous loss of ARX and PAX4 genes promotes the δ-cell fate specification at the expense of α- and β-cells (Collombat et al., 2005, Collombat et al., 2009).

ARX inactivation could induce the α-to-β-cell reprogramming in pancreatic progenitor cells or mature α-cells at any developmental or age stages, including embryonic, neonatal or mature stages (Collombat et al., 2003, Courtney et al., 2013, Hancock et al., 2010). The α-cell identity appears through an intermediate bihormonal state and is transformed into β-cell in the final (Wilcox et al., 2013b). The α-to-β-cell conversion induced by ARX seems not to stop until all α-cells change to β-cells totally (Courtney et al., 2013). The ectopic expression of ARX induces in progenitor or mature β-cells leads to a loss of the β-cell identity and a dramatic increase in a number of α- and γ-cells (Collombat et al., 2007), which means ARX is probably deactivated in β-cell. Thus how to suppress activeness of ARX in β-cell, and how to activate the function of ARX in β-cell has become a hotspot, and the epigenetics study gives us some inspiration (van der Meulen and Huising, 2015). It is found that methylation of ARX plays critical role in determining the identity of different pancreatic endocrine cells, and ARX is hypomethylated in α-cell and methylated in β-cell (Dhawan et al., 2011). In differentiated β-cells, the ARX promoter is highly methylated and this is facilitated by the de novo DNA methyltransferase Dnmt3a (Chen and Chan, 2014, Papizan et al., 2011). Transcription factor Nkx2.2 binds the hypermethylated promoter of ARX in a complex with Dnmt3a and preferentially recruits Tle3 and HDAC1 to repress ARX (Papizan et al., 2011, Schaffer et al., 2013). In the proliferation and regeneration of β-cells, the ARX regulatory region maintains methylation status induced by another DNA methyltransferase Dnmt1 to prevent the decrease of DNA methylation in β-cell division (Dhawan et al., 2011, Nishiyama et al., 2016). Furthermore, methylated region of the ARX locus in β-cells is bound by the methyl-binding protein MeCP2, which recruits the HMT PRMT6 that mediates H3R2 methylation, resulting in repression of ARX (Dhawan et al., 2011), which is shown in Fig. 4. The following experiments demonstrate that Deficiency of Dnmt1 or Dnmt3a both lead to the β-to-α-cell conversion (Dhawan et al., 2011, Papizan et al., 2011). However, it is still unknown that which β-cell specific factor preferential recruit Dnmt3a in this process because Dnmt3a is also expressed in both α- and β-cell (Papizan et al., 2011). The different pathway of Dnmt3a in α- and β-cell should be next research focus in the future. Moreover, another experiment showed that within 3 months of Dnmt1 and Arx loss, lineage tracing and single-cell RNA sequencing revealed extensive α cell conversion into progeny resembling native β cells, which indicated that pathways regulated by Arx and Dnmt1 that were sufficient for achieving targeted generation of β cells from adult pancreatic α cells (Chakravarthy et al., 2017).

In addition, pancreatic G-cell which could secret hormone gastrin has been recently identified in embryonic islet as 6th endocrine cell type, and its formation depends on ARX. Relevant data suggest that 70% reduction in the levels of gastrin mRNA in embryos of mice deficient for ARX (Suissa et al., 2013). Since G cell has
just been discovered in pancreas, correlative research reports are few and the relationship between gastrin and ARX shall be clarified by further study.

Apoptosis

Recently some studies show that several ARX mutations could lead to apoptosis of a-cells (Wilcox et al., 2013a, Xu et al., 2013) which is originally proposed in the research on hyperplasia of α-cells induced by absolute or relative deficiency of glucagon secretion in vivo(Hayashi et al., 2009). It is found that expression of ARX mRNA is extremely up-regulated in the huge pancrease of mice lacked pro-glucagon gene, and this compensatory hyperemia could be depressed when ARX gene is mutated in vivo, including GCG7 and P335L mutations (Hayashi, 2011, Hayashi et al., 2009, Xu et al., 2013). In this case the reduction of pancreatic a-cell could be due to an increasing a-cell apoptosis, while the β-cell mass remain no change and some β-cell specific transcription factors show no significantly up-regulated.

Similar results are obtained by analysis on the pancreas of patients with ARX mutations. Itoh M et al., firstly revealed the abnormal distribution of the component cells of Langerhans islets and the exocrine system of two male XLAG patients with ARX mutation in 2010. Both of them died before the second year with various mutation or deletion of nucleotide changes in ARX gene. Abnormal gene sequences lead to severe clinical presentations including relatively smaller pancrease followed by increase of fibrous interstitium and small islets of Langerhans showed deficient of a- and γ-cells(Itoh et al., 2010). However, the number of β-, δ- and ε-cells are not significant reduced compared with those of age-matched controls, and the transcription factor Brn4 and Pax6 which both bind to the pro-glucagon gene promoter is not detected in the pancreas with Arx mutations(Gosmain et al., 2011, Itoh et al., 2010).

With the help of those animal experimental and clinical results, it is obviously demonstrated that a different pathway is regulated and affected. For instance, Rb and E2f1 pathway is closely associated with tumor suppressor p53 and p16 which have a significant role in apoptosis, autophagy and senescence(James and Peters, 2000, Laine and Westerman, 2014, Madan et al., 2012, Udayakumar et al., 2010). It is possibility that the binding capacity is changed by site-directed mutagenesis in exon2 of ARX gene, which results in up-regulation of apoptosis factors. Naturally it is a conjecture or hypothesis based on known pathway, the definite mechanism should be verified by detailed experimental analysis.

Expectation and conclusion

ARX gene has been in our sight for a time. According to the previous research on pancreas, it is considered as one of determined factors for early specification of a-cell and maintenance of a-cell identity, but not directly involved in glucagon expression, and common used as a specific a-cell biomarker to analysis the change and fate of endocrine cell types(Rezania et al., 2011, Riedel et al., 2012).

Current research into diabetes treatment, especially type1 diabetes caused by the loss of β-cells and insulin secreted, is focus on generating replacement cells from other sources, including stem cells, progenitor cells and differentiated cells(Courtney et al., 2011). ARX inactivation is expected to induce stem cell or other mature pancreatic endocrine cell in to insulin-producing cell as an essential factor(Pearl and Horb, 2008). Surprisingly, the experiment in vitro suggests that ARX inhibition does not have obvious effective on the transdifferentiation into β-cells in differentiated human embryonic stem cells using 33-day and7-satges protocol or expanded α-cells treated with a combination of soluble factors(Gage et al., 2015). However, misactivation of ARX inhibits the redifferentiation of ex-vivo expansion of β-cells, elevates insulin mRNA levels and increases the productivity of insulin-positive cells, which suggests ARX blocking could be an effective approach of facilitate the generation of abundant β-cells under defined conditions(Friedman-Mazursky et al., 2016).

Several lines of evidence indicate that α-cells could be other potential progenitors of β-cells in vivo(Habener and Stanojevic, 2013). It is well known that β-cell self-replication can be used to supplement relative and absolute deficiency of insulin-producing cells in β-cell injury or diabetes model, however, this system would failure if the ablation of β-cells were extreme(Bouwens and Rooman, 2005). In this case, the regeneration of new β-cells mainly depends on the directly transdifferentiation from preexisting α-cells(Habener and Stanojevic, 2013). Recent study shows that a prompt expansion of β-cells occurred in mice with special ablation of ARX in mature α-cells. It can be inferred that the decrease of glucagon signaling induces pancreatic duct cells re-express NGN3 and continuously differentiate into α-cell, however, these neogenic α-cells are convert into β-cells gradually owing to the deficiency of ARX(Courtney et al., 2013). These evidences suggest that ARX could be used in generating β-cells as a potential target in diabetes treatment.

Still a bit not allow to ignore, the security of ARX suppression should be considered as a vital dimension. Although ARX-null mice die within 2 days after birth, its role in pancreas is not the main cause of mortality(Collombat et al., 2003, Hancock et al., 2010). The decrease of α-cells caused by disruption of ARX couldn’t lead to severe metabolism physiologically changes in maturity individuals(Hancock et al., 2010). This conclusion is consistent with previous findings that the blood glucose level and life span maintain normal in the mice deficient for pro-glucagon gene(Hancock et al., 2010, Hayashi et al., 2009). Furthermore, the second worry is fatty liver owing to the absence of serum glucagon level (Hancock et al., 2010). In fact, different mutation type and mutation site have important impact on the α-cell fate, and some evidences indicate that precious few α-cells 98% α-cell ablation could adjust and maintain normal serum glucagon level over time(Thorel et al., 2011). There are reasons to believe that this fatty liver case could be avoided. Taken together, it can be speculated that ARX is a reliable and safe target for diabetes treatment.

Concluding remarks

Although the molecular pathway and pathogenesis still remain puzzling, what’s clear is that the role of ARX is vital to the endocrine cell fate, especially α-cell identity and survival. With the technical development and the research being unceasingly thorough, there are more profound recognitions to the function of ARX in endocrine pancreas. These progresses on experimental biology and clinical medicine have been of great benefit for scientific advancement and treating disease. Based on the existent
knowledge and experiences, the utilization of ARX is worth looking forward in diabetes therapy.

Conflict of interest
The authors declare they have no competing interests or other interests that might be perceived to influence the results and discussion reported in this paper.

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