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THE ROLES OF MICRO RNA IN PANCREAS DEVELOPMENT AND REGENERATION

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Presence of sufficient number of functional glucose responsive β-cells is indispensable for normal glucose homeostasis. Diabetes mellitus is a chronic disease associated with loss or reduction of β-cell mass and not β-cell mass. MicroRNAs are small non-coding RNAs that are involved in different biological processes including development, cell proliferation, stress response, and tumor pathogenesis. MicroRNAs fine-tune the gene expression level post-transcriptionally either by mRNA degradation or translational repression. In the past few years, several miRNAs have been introduced as new critical players for pancreas development, function, and regeneration. Deregulation of several microRNAs is found in animal models of diabetes, as well as in diabetic patients. Therefore, it is essential to understand the roles of these microRNAs in β-cell generation and physiology, as well as the biological consequences of their functional impairment. ***Biomed Rev*** **2013; 24: 57-65**

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INTRODUCTION

The pancreas consists of two different compartments, the exocrine and the endocrine cell populations. The exocrine compartment consists of acinar and duct cells, which are involved in secretion of digestive enzymes (acinar cells) and their transport to the duodenum (duct cells). The endocrine pancreas is organized in the so-called 'islets of Langerhans', functional units consisting of α-, β-, δ-, PP- and ε-cells expressing glucagon, insulin, somatostatin, pancreatic polypeptide and ghrelin, respectively (1).

Loss or reduction of β-cells mass and not β-cell mass is the hallmark of both type 1 and 2 diabetes mellitus, a disease with long-term severe complications like amputation, diabetic coma, and cardiovascular diseases. The final approach for diabetes treatment should lead to exogenous insulin-independent

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therapy. Currently, the most common strategy toward this goal is replacement of β-cells using islet-transplants from donor pancreata. The main drawback here is the limitation of donor islets. *In vitro* generation of β-cells is a potentially reliable alternative method that can overcome the resource limitation of islet transplantation (2,3). To generate fully functional β-cells *in vitro* it is necessary to understand the mechanisms underlying pancreas and specifically islet and β-cell development. While a battery of transcription factors and signaling pathways are investigated in this context and their function in pancreas development become more and more clear (4-13), recent findings have also shown that miRNAs play fundamental roles in pancreas and β-cells development.

Caenorhbditis elegans was the first organism, in which the importance of miRNAs was discovered (14). In general, miRNAs act as negative regulators of gene expression. MiRNAs are first transcribed as pri-miRNAs, and then cleaved by Drosha RNAse III endonuclease to generate the pre-miRNAs that are about 60-70 nucleotides long (15). The pre-miRNAs are transported to the cytoplasm, where they are processed by Dicer to generate the miRNA/miRNA* double strand (16). The mature miRNA will be incorporated into the miRNA-induced silencing complex (miRISC) that finally binds to the 3'UTR of their target sequence, resulting in either target mRNA degradation or translational repression (15,16).

In the past few years, the importance of miRNAs in regulation of different developmental processes has become more and more evident. Several studies indicate that these micro-players are implicated in pancreas development as well as type 1, and type 2 diabetes (17-19). In this review, we discuss the multiple roles that miRNAs may play during endocrine pancreas development and regeneration (Figure 1).

miRNAs IN PANCREAS DEVELOPMENT

Analyses of pancreas-specific Dicer knockout mice revealed that miRNAs are essential for pancreas development and β-cell formation (20). Thus, the depletion of Dicer, the enzyme that is necessary for biosynthesis of microRNAs, in Pdx1-positive pancreatic progenitors, is accompanied with severe defects of pancreatic tissue, especially due to the loss of endocrine cells. The most affected cell type was the β-cell, exhibiting 94 % reduction compared to wild type littermates (20). Furthermore, the early loss of dicer in pancreatic progenitors leads to upregulation in Hes1 expression, and resulting in decreased numbers of Ngn3-positive endocrine cells. In addition, following inactivation of Dicer in β-cells, reduction of Insulin transcription, and content, as well as hyperglycemia were noticed (21). Moreover, conditional knockout of Dicer in β-cells provokes disturbance in insulin secretion, as well as progressive depletion of insulin producing cells, suggesting a role for miRNAs in β-cell survival (22,23). In the pancreata of these mice, the cell adhesion molecule E-cadherin is also deregulated and as a consequence, the islet architecture is disrupted (22). Recently, analyses of Dicer-deficient endocrine progenitors indicate that following the first neonatal days hormone expression is abolished. Instead, the expression of neuronal genes, such as tyrosine hydroxylase or stathmin-like 2 is activated (24). Hence, miRNAs appear to promote the endocrine cell fate by repressing neuronal gene activity in endocrine progenitors. Overall, these findings illustrate the important role of miRNAs in β-cell development and maintenance.

To figure out which specific miRNAs are involved in pancreas development and/or β-cell survival, the expression profile of miRNAs in the developing pancreas as well as α- and β -cells of different species has been investigated (25-30). One of the most studied miRNAs during pancreas development is miR-375. MiR-375 is highly expressed in pancreatic islet (29,31) and was shown to regulate insulin secretion in primary islet explants, as well as in MIN6 β-cells by inhibiting mytropin (Mtpn) (29). Knockdown of miR-375 in zebrafish using morpholinos resulted in a dramatic malformation of endocrine cells in the developing embryo at 48 and 72 hours post fertilization (hpf). The knockdown embryos show scattered insulin-, glucagon-, PP- and SST-positive cells while the WT embryos exhibit single clusters of hormone-expressing cells (28). At 24 hpf, the expression of the pancreatic hormones is still normal in the knockdown embryos, suggesting a more prominent role for miR-375 in maintenance of endocrine cell fate and tissue identity than in early endocrine cell development (28). Analysis of mice lacking miR-375 support these findings, and knockout animals display a reduced number of β-cells, whereas the pancreatic α-cell content is increased. Accordingly, these mice suffer from hyperglycemia that is mainly caused by hyperglucagonemia (32). In summary, miR-375 appears to target genes that are necessary for the control of endocrine cell identity and/or proliferation. However, direct targets of miR-375 remain to be identified. It should be noted that miR-375 is not only important for pancreas development, but it also required for maintaining β-cell physiology. Silencing of miR-375 in MIN6 and mouse primary β-cells is accompanied with increased glucose-stimulated insulin secretion (29).

MiR-7 is also highly expressed during pancreas develop-

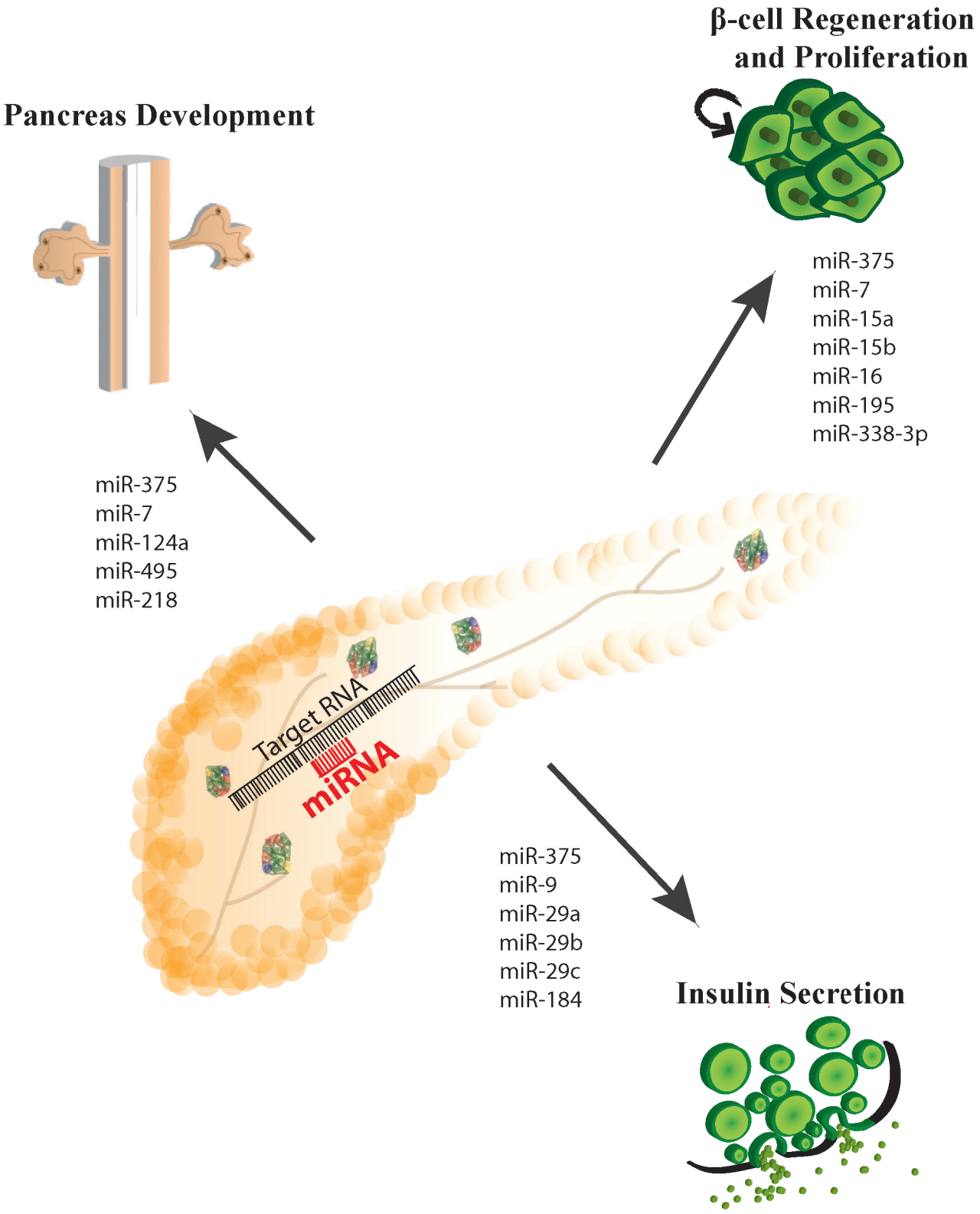

Figure 1: *miRNAs involved in pancreas development, β-cell regeneration, and insulin secretion.*

ment in human and mice (33-36). *In situ* hybridization studies showed that miR-7 is only expressed in endocrine but not in exocrine pancreatic cells, suggesting a possible role of miR-7 in endocrine development (33, 35). MiR-7 is already detected in Ngn3-positive endocrine progenitors in mice (35), but not in humans (33). Knockdown of miR-7 in mice provokes a reduction in insulin production and decreased β-cell content, while the transcript level of the other endocrine hormones (glucagon, somatostatin and pancreatic polypeptide) remains unaffected (36). These results are in contrast to a more recent study where silencing of miR-7 in primary pancreatic explants is accompanied with increased number of both insulin and glucagon expressing cells (35). These divergent findings might be related to the choice of the experimental systems, performed *in vivo* (36) and *ex vivo* (35), and at different developmental stages. This may indicate that miR-7 has distinct roles at different stages of pancreatic endocrine genesis. MiR-7 might promote differentiation, and/or survival of endocrine cell survival. Overexpression of miR-7 in *ex vivo* pancreatic explants provokes a phenotype that is reminiscent of heterozygous *Pax6*-deficient mice, where amongst others the levels of insulin and MafB are down regulated (35). Moreover the level of Pax6 transcripts was significantly reduced (35). MiR-7 overexpression in Pdx-1 progenitors in mice corroborates the findings from explant culture. Further, molecular analyses provide evidence that miR-7 controls and refines Pax6 levels in the endocrine pancreas (35).

MiR-124a is expressed in the early developing pancreas and appears to regulate Foxa2 expression in pancreatic MIN6 β-cells (37). Foxa2 itself is involved in pancreas development by regulating Pdx1 expression (38). Following knockdown of miR-124a in MIN6 cells Pdx1 and Insulin levels are augmented, while its overexpression results in an opposite alteration. MiR-124a was also suggested to modulate Insulin secretion in MIN6 cells (37). Notwithstanding, miR-124a function in pancreas development remains obscure. In addition to the discussed miRNAs miR-495 and miR-218 were also suggested to be involved in pancreas genesis. They target the 3'-UTR region of HNF-6, which is an important transcription factor required for correct timing of pancreas specification (39, 40).

Although all of the discussed studies clearly demonstrate the importance for miRNAs during pancreas development, their proper function and targeting pathways in this context remain still unclear. Therefore, it is crucial to investigate their targets to get further insights into the gene networks controlling pancreas and especially β-cell development.

miRNAs IN INSULIN SECRETION

As mentioned above, one of the direct targets of miR-375 is myotropin (Mtpn). Mtpn is involved in insulin secretion (29). Overexpression and inhibition of miR-375 in MIN6 cell leads to down and enhanced insulin secretion, respectively. Knockdown of Mtpn itself correlates with miR-375 forced expression (29). How Mtpn exactly regulates Insulin secretion remains unclear and needs further investigation.

As already mentioned, miR-124a regulates Foxa2 expression in the insulinoma pancreatic b-cell line MIN6. As Foxa2 plays prominent role in Insulin secretion and glucose homeostasis (41), it is conceivable to speculate that miR-124a might also be involved in Insulin secretion pathway. Accordingly, it was shown that miR-124a and miR-96 modulate protein levels of SNAP25, Rab3A, Rab27A and Noc2, which are all known to be engaged in the process of insulin secretion (42).

Overexpression of miR-9 in β-cells and the pancreatic β-cell line Ins-1 leads to impairment of glucose-stimulated insulin secretion through regulation of onecut 2 (43). Onecut 2 modulates granuphilin expression, which plays important role in exocytosis. Overexpression of miR-9 reduces onecut 2 expression, which in turn causes accumulation of higher granuphilin level that leads to impaired exocytosis (43). These results demonstrate that miR-9 negatively regulates insulin secretion.

The miR-29 gene family consists of three paralogs: miR-29a, miR-29b and miR-29c. Raising the level of one of these miRNAs in MIN6, Ins-1E or primary mouse islet cells leads to reduction of glucose-induced insulin secretion (44,45). As shown for miR-9, the expression level of onecut 2 is decreased upon miR-29 overexpression, possibly justifying the observed phenotype in glucose-stimulated Insulin secretion (45). Moreover miR-29a directly targets syntaxin-1a, another protein implicated in insulin exocytosis (46).

Another miRNA involved in insulin secretion is miR-184, which is enriched in human pancreatic islets (47). MiR-184 regulates glucose-stimulated insulin secretion through repressing Scl2a22 (48), which plays an essential role in glucose-stimulated insulin secretion of β-cells (49).

Overall it is clear that distinct miRNAs have different targets in the Insulin secretion network. In order to fully understand the underlying mechanisms that make a functional glucose-responsive β-cell, it is necessary to uncover the exact function of miRNAs that regulate this process by fine-tuning the level of distinct modulators.

miRNAs IN β-CELL REGENERATION

Proliferation of β-cells has been described in adult rodents in response to physiological increased metabolic demands in obesity, and pregnancy. β-cell regeneration was shown to occur following pathophysiological conditions caused by pancreatic injury or disease (50,51). Several animal models are used to study the mechanisms underlying β-cell regeneration, and it is now believed that the regenerative capacity, and the mechanisms through which it takes place, are highly dependent on the extent of β-cell loss, and type of injury. Three main mechanisms were suggested to contribute to β-cell regeneration in adult rodents. In most of studies, adaptive β-cell proliferation predominantly represents the major source for increased β-cell mass upon β-cell depletion (52-54). Furthermore, higher metabolic demands during pregnancy and insulin resistance can also induce β-cell proliferation, and leads to increased β-cell mass, which then can compensate for the lower peripheral sensitivity (55,56). Some other studies have shown that β-cells can be differentiated from progenitor cells in pancreatic duct, as they do during embryonic development (57-60). Recently, some groups have revealed the potential of α-cells to switch their fate and transdifferentiate to β-cells in extreme β-cell loss condition (61) (62). Emerging evidences suggest that miRNA can mediate the responses to different types of cellular stresses through post-transcriptional modification of mRNAs, which leads to changes in gene expression patterns and thereby activation and inhibition of different biological processes in the cells (63). For example, miRNAs induced by inflammation can guide the cell to mediate the inflammatory responses and to switch them off later (64). In another interesting example, an endothelial-specific miRNA mediates stress-induced proliferation, angiogenesis, and wound repair (65).

There are few reports on miRNAs regulating β-cell stress response and regeneration. MiR-375 is an islet-specific miRNA that has an essential role in maintenance of α- and β-cell mass, as discussed above. Additionally, miR-375 is increased in the islet of ob/ob mice and its deficiency impairs both basal proliferation and the adaptive proliferation of β-cells induced by Insulin resistance and obesity. Although under normal conditions, minor reduction in β-cell mass did not have a severe impact on plasma Insulin level, lack of compensatory proliferation in miR-375 KO Insulin resistant obese mice leads to a drastic decrease in β-cell mass and Insulin level compared to the ob/ob littermates. By comparing the gene expression pattern of pancreatic islets from miR-375 KO and wildtype littermates to find potential miRNA targets among the genes upregulated in KO islet, it was concluded that miR-375 regulates a cluster of genes that are negatively regulating proliferation- and growth-related pathways. Cadm1, Rasd1, Eef1e1, C1qbp, Aifm1, Rgs16, Cav1, Id3, Smarca2, HuD, and Nnat are the potential target genes that were upregulated in miR-375 knockout islets (32). It is also shown that miR-375 can inhibit tumor cell growth in different carcinoma cell lines (66,67) and its expression is downregulated in pancreatic cancer (68).

Another abundant miRNA in islets is miR-7 (25,33), which was recently shown to target different components of mTOR signaling pathway, and thereby negatively regulates β-cell proliferation in mouse and human. The mammalian target of rapamycin (mTOR) is a kinase that forms two distinct complexes known as mTORC1 and mTORC2 (69). mTOR signaling pathway links the external and internal signaling pathway to modulation of cell growth and proliferation, cell survival, and metabolism. mTOR is mainly regulated by nutrients and growth factors and initiates the proliferative or anti-proliferative responses based on the growth-promoting or growth-suppressing signals (70, 71). Constitutive activation of mTORC1 in pancreatic β-cells leads to increased β-cell proliferation, β-cell mass, and Insulin secretion in the first phase. This is then followed by increased β-cell apoptosis and hyperglycemia due to cellular exhaustion (72,73). Consistently, loss of mTORC2 results in decreased β-cell mass and proliferation that eventually causes hyperglycemia and glucose intolerance (71,74). These observations clearly demonstrate that the level of several components of mTOR signaling pathway should be finely controlled at different cellular status, to keep a healthy balance between proliferation and survival.

As mentioned above, self-replication of pre-existing β-cells is the major mechanism replenishing β-cell mass in adult pancreas. But, there are some evidences showing that β-cell regeneration induced by certain type of damage and injury might recapitulate the developmental events in embryonic pancreas (51). For example, many transcription factors that play essential roles in pancreas development such as Foxa2, Pdx1 and also Ngn3 that is required for endocrine lineage specification, are reactivated after 70% pancreatectomy or pancreatic duct ligation. In some cases, Ngn3 expressing cells were detected in the duct epithelium and they also seemed to contribute to the newly formed β-cell population (60,75). Other studies have challenged the contribution of Ngn3 expressing cells to β-cell lineage (76,77). Importantly, further characterization of expression profile of regenerating pancreata suggested that Ngn3

mRNA level is post-transcriptionally regulated by miR-15a, miR-15b, miR-16 and miR-195, which are highly up-regulated in regenerating pancreas (78). Such mechanism might limit or suppress β-cell neogenesis via endocrine progenitors in adult life. It should be noted that the promoter-based strategies used for monitoring the re-activation of Ngn3 can only represent the promoter activation and mRNA expression (60,75) and do not take into account the miRNA-mediated post-transcription regulation imposed on Ngn3 transcripts. This might explain the controversial observations in different studies.

In a recent study, Jacovetti *et al* (80) have identified a set of miRNAs that are differentially expressed in the islets of pregnant rats. MiR-218, miR-338-3p, and miR-874 are found to be significantly downregulated at day 14 of gestation in pregnant rats compared to the age-matched non-pregnant females. In the same animals, miR-144 and miR-451 levels are significantly upregulated. Interestingly, they have observed that downregulation of miR-338-3p increases proliferation rate of INS832/13 cells and dissociated rat β-cells, but not human β-cells. Additionally, they have shown that reduction of miR-338-3p or increased level of miR-451 can protect cultured rat and human islet cells against pro-apoptotic effects exerted by exposure to pro-inflammatory cytokines. Further investigations showed that miR-338-3p level is also reduced in Insulin resistant db/db and HFD-fed mice. It was observed that treatment of rat and human islets with incretin hormone glucagon-like peptide 1 analog, Exendin-4, or pregnancy associated hormone17-β estradiol (79) decreases miR-338-3p level. They observed that the inhibition of miR-338-3p resembles the effect of GLP1 on β- cells and upregulates Igf1r and Irs2 expression. So, they hypothesized that miR-338-3p downregulation is associated with the compensatory β-cell mass expansion, and estrogens and incretin hormones mediate it (80).

CONCLUSION

Deregulated expression of miRNAs has been associated with many pathological situations and diseases. In many cases, it is not clear if the alteration in miRNA expression is in fact the primary cause or the subsequent effect of the disease. As discussed above, it appears that miRNAs may control various important aspects of β-cell development, function, and regeneration (Fig. 1). It should be noted that maintenance of homeostatic condition is a result of interplay of several genes, and miRNAs fine-tuning their levels at different states. Any imbalance in the level of these players could lead to development of complex diseases such as type 1 and type 2 diabetes. Therefore, getting more insights into the molecular mechanisms and linking the different contributors of these gene-miRNA networks is of fundamental importance for developing novel therapeutic approaches for diabetes. Finally, it is interesting to note that recent studies have highlighted the presence of miRNAs in body fluids and represented them as a new type of biomarkers for early diagnosis of diseases (81-83).

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