Chapter 5
MicroRNAs as Engineering Targets: Pathway Manipulation to Impact Bioprocess Phenotypes

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Abstract Chinese hamster ovary (CHO) cells are the primary mammalian culture system used for recombinant protein production; therefore there are continuous research and development efforts to improve cell production capabilities by both genetic modification and process optimization strategies. The genetic modifications are used to increase specific growth rate, to reduce apoptosis and to improve nutrients utilization. Since altering the expression of a single gene or even a single pathway may not be sufficient to produce desirable phenotypes, regulation of global gene expression may be a better approach for pathway engineering in CHO cells. miRNA(s) were found to be global regulators of gene expression with the ability to simultaneously alter multiple cellular pathways such as cell growth, apoptosis, stress resistance, metabolism and protein secretion. Therefore, modifications of miRNA expression profiles may facilitate the design of high-producing CHO cells. Recent advances in transfection techniques allow the insertion of miRNA mimics or inhibitors into CHO cells at specific stages of the bioprocess. Unlike traditional engineering approaches, manipulation of miRNA expression profiles does not burden the translational machinery of the cell and therefore, cellular metabolic resources are allocated to recombinant protein production. In this chapter we highlight the industrially-relevant pathways, report on miRNA involvement in their regulation, discuss how these miRNAs can be used to improve performance of CHO cells for industrial applications and propose specific miRNA candidates for CHO cell engineering.

Keywords Apoptosis · Mammalian cells · miRNA · Protein expression

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5.1 Introduction

5.1.1 Potential Role of MicroRNAs in Improving CHO Cells Suitable for Large-Scale Production of Biologicals

Chinese hamster ovary cells are the primary mammalian culture system used for production of biopharmaceuticals. This is attributed to their robustness in a bioreactor and their ability to produce properly folded recombinant proteins with human-like posttranslational-modifications which is important for maintaining their biological activity (Barron et al. 2011b; Druz et al. 2011; Kramer et al. 2010; Muller et al. 2008).

Mammalian-based systems for biological production are required to produce high quantities of product and therefore need to meet process-related requirements that place high demands on CHO cells to generate suitable product titers and quality (Dinnis and James 2005). Improving and predicting the performance of CHO cells in bioreactors is important for enhancement of cell specific productivity (Clarke et al. 2011).

Limited growth capacities and low tolerance to different environmental conditions in bioreactors (nutrient and growth factor depletion, shear and oxidative stresses, metabolite accumulation, pH, osmolality and hypoxia) affect cell productivity. It is possible that these conditions cause apoptosis, reducing the yield and the quality of the produced proteins (Lim et al. 2010). Generation of stress-resistant CHO cell lines suitable for efficient production of various biologicals is therefore important for the biopharmaceutical industry. Additional desired characteristics of high-producing CHO cells are rapid growth, long term genomic stability and protein secretion capacity.

The discovery of microRNAs (miRNAs) offers an opportunity to improve the performance of CHO cells in an industrial-scale bioprocess. It is predicted that nearly half of the proteins may be affected by miRNAs (Lewis et al. 2005) which are more abundant than transcription factors. miRNAs have been shown to be global regulators of gene expression affecting almost all essential cellular processes and functions. So far, it has been found that miRNAs are involved in cell development, differentiation, metabolism and proliferation. miRNA expression profiles can also be explored as biomarkers for desired properties such as resistance to various stresses, growth and clonal stability and/or engineering targets (Barron et al. 2011a; Druz et al. 2011).

The potential of miRNAs utilization for mammalian cell engineering was highlighted by analyzing miR expression profiles under various physiological conditions (Druz et al. 2011; Gammell 2007). Each miRNA has the potential to post-trascriptionally affect more than 100 targets and concurrently influence several interlinked pathways or multiple points of the same pathway in the cell, which may facilitate creating industrially-relevant phenotypes (Barron et al. 2011a). One of the key advantages for utilizing miRNAs rather than regulatory proteins such as transcriptional factors or kinases is the fact that they do not burden the translational machinery and therefore reduce the metabolic load on the host cells. As a result, cellular metabolic resources will be better allocated to the production of

recombinant protein, leading to increase of cell specific productivity. In addition, the cell can respond faster to miRNA modulations, either naturally or by introduction of exogenous mimics or inhibitors since there is no need for a translation step (Barron et al. 2011a; Muller et al. 2008).

Novel large scale transfection techniques allow relatively easy insertion of miRNA mimics or inhibitors into CHO cells at some stage of the bioprocess (Barron et al. 2011a). Also, some efficient miRNA expression systems which contain drug-inducible or heat-shock-inducible promoters that control the timing and expression levels of chosen miRNAs have been recently reported (Weber and Fussenegger 2007; Yang and Paschen 2008). Therefore, the investigation of miR expression profiles in CHO cells under different physiological conditions will facilitate detection of specific miRNAs that can be manipulated to obtain desired properties in CHO cells. Alteration of those miRNA expression profiles will introduce another dimension to genetic engineering of CHO cell to generate more robust CHO cell lines better suitable to production of biologics (Druz et al. 2011).

5.1.2 Current Strategies for CHO Cell Engineering

There is currently an effort to improve CHO cells' performance and productivity through better understanding of the physiology of the cells and their response to the physical and chemical environment in the bioreactor.

Current process optimization techniques focus on increasing integrated viable cell density (IVCD) in the bioreactor by enhancing the specific growth rate, reducing apoptosis, redirecting metabolic pathways to lower accumulation of toxic byproducts, and increasing nutrient utilization efficiency (Druz et al. 2011; Muller et al. 2008; Chen et al. 2001; Elias et al. 2003; Irani et al. 2002). Most cell line improvement efforts are based on media manipulation or gene engineering approaches by random integration in the cell. However, newly developed methods such as targeted homologous integration, RNA interference techniques and zinc-finger nuclease deletions should be evaluated as approaches for cell improvements (Kramer et al. 2010).

One of the main obstacles that researchers face while attempting to alter the CHO cells performance is the complexity of the intended phenotype such as rapid cell growth and resistance to various stresses in the bioreactor. Altering the expression of a single gene or protein or even a single pathway may not be sufficient to produce the desirable phenotype (Barron et al. 2011b). Therefore in some instances, cells have been "double engineered" to reach higher growth rates and increase apoptosis resistance (Fussenegger and Bailey 1998; Ifandi and Al-Rubeai 2005). It should be noted however that over-engineering of the cells may affect growth due to high metabolic load on the cells (Yallop and Svendsen 2001).

A comprehensive understanding of cellular pathway control and interaction is required for developing strategies to globally affect gene expression profiles for the purpose of engineering CHO cells with improved production properties (Dinnis and James 2005). Some success has been shown with the modification of the global gene expression levels through over-expression of transcription factors and artificial

Zn-finger proteins (ZFP). Over-expression of ATF4 and Xbp1 transcription factors and the artificial ZFP-TF derived from a synthetic library increased recombinant protein production capacity of CHO cells (Kwon et al. 2006; Ohya et al. 2008; Tigges and Fussenegger 2006). Dinnis and James (2005) suggested an approach based on imitating the molecular events of differentiation of B-lymphocytes into high producing plasma cells. This inverse engineering strategy enabled the synchronized expansion and maintenance of high levels of metabolic and secretory cellular machinery to increase cell-specific production rate in CHO cells.

Below we describe the industrially-relevant phenotypes and pathways with the existing strategies to improve performance of CHO cells, together with reports on miRNA involvement in the control of respective pathways. We concentrate on the possible manipulation of selected microRNAs in CHO cells to globally affect gene regulation and improve cell performance.

5.2 Applications of MicroRNA for Pathways Engineering in CHO Cells

5.2.1 Engineering of Growth and Growth Arrest

Volumetric production of mammalian cell culture is a function of cell specific productivity and IVC. Higher growth rates increase the rate of biomass accumulation and the number of viable cells, but suppressing cell growth can increase cell specific productivity by redirecting the metabolic energy from growth to recombinant protein production and secretion (Muller et al. 2008; Dinnis and James 2005). This conflicting relationship is managed by a "biphasic" cell culture strategy where in the first phase the cell growth is not limited, and in the second phase the cells growth is arrested to form high-producing cells. The challenge in this approach is to find a way to arrest cell division without promoting cell death or interfering with recombinant protein production (Dinnis and James 2005).

Techniques to induce growth arrest include manipulation of growth parameters (temperature, pH, hyperosmotic pressure), alteration of extracellular environment by adding specific metabolites or DNA synthesis inhibitors, and addition of cell-cycle regulators such as nucleotides or nucleosides (Altamirano et al. 2001; Bi et al. 2004; Carvalhal et al. 2003; Kim and Lee 2002). Each of these methods may affect multiple cellular processes simultaneously. For example, reduction of culture temperature was reported to suppress cell growth in a biphasic process which increased product titer in CHO cells; at the same time, lowered temperature induces changes in gene expression, protein phosphorylation, nucleotide pools, and consequently a reduction in cell metabolism (Kaufmann et al. 1999; Yoon et al. 2003). In addition, the effects of reduced temperature may be cell or product-specific and cannot be generalized (Dinnis and James 2005; Yoon et al. 2003).

Genetic control of cell cycle progression is another strategy to induce growth arrest. This can be done by activation of intrinsic cell cycle modulators including

genes encoding the $p21^{CIP1}$ and $p27^{KIP1}$ cyclin E-dependant kinase cdk2 inhibitors which cause arrest of the cell cycle at G1 phase (Fussenegger and Bailey 1998). This strategy of cell cycle arrest may also have its own limitations since it is difficult to engineer higher cell growth rates and growth arrest simultaneously. Besides, the improvement of cell specific productivity resulting from this approach may also be cell and product-specific (Dinnis and James 2005).

A microRNA-based strategy is a possible alternative to the existing methods of manipulating cell growth and arrest. There are reports on miRNA involvement in cell proliferation and growth by targeting essential genes of cell cycle regulation. The function of several members of miRNA 17–92 polycistronic cluster was investigated. Inhibition of miR-18a with antagomirs decreased cell proliferation (Scherr et al. 2007) and miR-17–5p and miR-20a negatively regulated the expression of E2F1 protein (which, when up-regulated promotes cell cycle progression and overrides the growth arrest), (O'Donnell et al. 2005). Another study showed that the inhibition of miR-21 and miR-24 resulted in a significant increase in mammalian cell growth (Cheng et al. 2005). The growth-inhibitory properties of miR-21 and miR-24 were also confirmed by their up-regulation during the stationary phase and after a temperature shift when compared to their levels during the exponential growth phase (Gammel 2007). It was suggested that miR-24 inhibits the progression thorough G1 phase and transition to S phase of the cell cycle by targeting E2F2 and MYC, and may also regulate DNA repair (Lal et al. 2009).

Several miRNAs have been reported to act as regulators of cell growth cycle. (1) miR-125b was reported to support cell growth in liver cells, and its inhibition in human cancer cell lines caused decreased growth (Gammell 2007; Lee et al. 2005); (2) over expression of Let-7 suppressed growth in colon cancer and lung adenocarcinoma by targeting MYC and RAS gene expression, thus affecting the pathways controlled by these oncogenes (Akao et al. 2006; Johnson et al. 2005); (3) expression of miR-133 was found to be correlated with a higher skeletal muscle growth rate by repression of serum response factor (SRF) in cultured myoblasts (Chen et al. 2006); (4) the miR-34 family (miR-34 a, b, c) was found to target growth and growth-arrest related genes including CDK4,and CDK6, c-MYC, CREB, Notch1, E2F3 (Hermeking 2010).

Other miRNAs affect proliferation of mammalian cell lines: (1) miR-143 and miR-145 negatively affect cell proliferation by regulating genes of the mitogen-activated protein kinase (MAPK) family, MAPK7 and ERK5 (Esau et al. 2004); (2) miR-221 and miR-222 inhibit proliferation in hematopoietic progenitor cells lines by targeting the Kit gene, which encodes receptor protein with tyrosine-kinase activity (Felli et al. 2005); (3) miR-372 and miR-373 control proliferation by direct interference with cell cycle regulation, regardless of increased levels of the inhibitor $p21^{CIPI}$ and inhibition of CDK by p53; (4) over expression of miR-31 exhibits anti-proliferative effects in multiple cancer types by associating with the p53-mediated growth inhibitory pathway, and may trigger caspase activation and apoptosis by suppressing E2F2 gene (Creighton et al. 2010); (5) over expression of miR-7 inhibited growth by targeting epidermal growth factor receptor (EGFR) and Akt pathway in glioblastomas. Exogenous increase of miR-7 levels blocked CHO-K1 proliferation

and increased cell productivity, suggesting its possible role in CHO cells engineering via decoupling cell growth from growth arrest to enhance recombinant protein production (Barron et al. 2011a; Kefas et al. 2008).

The use of miRNAs for engineering CHO cells better suitable for growth and production needs to be evaluated carefully since their effects may be tissue or cell-specific (Gammel 2007). miR-21 and miR-24 have already been reported in CHO cells and seem to have growth-inhibitory properties (Gammel 2007). Recent work on sequencing the CHO microRNA transcriptome will serve as a valuable resource for identification of specific miRNAs involved in control of cell proliferation and cell cycle. Next-generation sequencing of miRNAs across different industrially-relevant conditions such as temperature shift and sodium butyrate treatment, helped to identify and annotate 387 mature miRNAs conserved in CHO including the members of miR 17–92 polycistronic cluster, miR-221/222 cluster, let-7 family, miR-34 family, miR-7, miR-125b, miR-143, and miR-31 (Hackl et al. 2011; Johnson et al. 2011). The sequencing of cDNA of miR 17–92 cluster validated targets confirmed the conservation of respective miRNA binding sites in 19 genes in CHO. The latter serves as a confirmation of the conserved biological role of this cluster in CHO cells (Hackl et al. 2011). Further studies of biological functionality of the reported CHO-specific miRNAs as compared to their role in other mammalian cells will facilitate identification of key miRNAs for growth and cell cycle engineering in CHO cell factories.

5.2.2 Engineering of Apoptosis-Resistant Cell Lines to Increase IVC

Apoptosis or programmed cell death (PCD) is a necessary physiological function in multicellular organisms that presents difficulty for maintaining high viable cell densities in mammalian bioprocess applications (Muller et al. 2008). Different stress conditions in bioreactors such as nutrient limitation, byproduct accumulation, shear and oxidative stresses, pH, osmolality and hypoxia can trigger apoptosis during CHO cell cultures. Onset of apoptosis results in a lowered IVC which affects product yield and properties (Druz et al. 2011; Gammel 2007). As a result, apoptosis prevention is one of the most investigated techniques in CHO cells engineering.

There are two main approaches to inhibit or slow the apoptotic cascade activation. The first involves the manipulation of the outer cellular environment by media supplementation with growth factors, limiting nutrients, and hydrolysates (Majors et al. 2007; Zanghi et al. 1999). The second employs genetic engineering methods to reallocate the tightly regulated balance of pro- and anti-apoptotic factors in favor of the anti-apoptotic proteins (Chiang and Sisk 2005; Lim et al. 2006; Wong et al. 2006). Anti-apoptotic proteins such as Bcl-x_L, Bcl-2 and Mcl-1 are known to protect the cells from apoptosis by maintaining the integrity of mitochondrial membrane. Exogenous expression of these proteins increases cell density and viability and protects the culture from apoptosis induction by different stimuli in mammalian cells (Fassnacht et al. 1999; Fussenegger et al. 2000; Mastrangelo et al. 2000;

Sauerwald et al. 2006; Tey et al. 2000). Also over-expression of viral homologs of bcl-2 such as E1B-19K was shown to reduce apoptosis in mammalian cell cultures (Figueroa et al. 2007; Mercille and Massie 1999; Sauerwald et al. 2002).

RNAi silencing techniques against pro-apoptotic genes have been successfully applied. Stable inhibition of Bax and Bak with shRNA vectors, and silencing of the apoptosis linked gene Alg-2 and the transcriptional factor Requiem in CHO cells delayed apoptosis, increased cell viability and improved interferon-γ production (Lim et al. 2006; Wong et al. 2006). Also, the simultaneous down-regulation of caspase-3 and caspase-7 expression improved cell viability and thrombopoietin production in CHO cells after sodium butyrate treatment (Sung et al. 2007).

miRNA involvement in apoptosis regulation was initially studied in peripheral blood cells of people diagnosed with chronic lymphocytic leukemia (CLL) where deletion of miR-15 and miR-16 genes was reported in the majority of patients (Calin et al. 2002). Later studies revealed that these miRNAs were promoting apoptosis in malignant B cells by targeting Bcl-2 expression at the post-transcriptional level (Cimmino et al. 2005). miR-21 (in addition to its involvement in cell growth regulation) was found to be up-regulated in several human cancers and was characterized as an oncogenic miR. Silencing of miR-21 expression in glioblastoma cells led to increased apoptosis by activation of caspase 3 and 7 (Chan et al. 2005; Meng et al. 2006; Si et al. 2007).

Cheng et al. have identified several miRNAs involved in apoptosis regulation by using large-scale antisense miRs inhibition. The inhibition of miR-1d, 7, 148, 204, 210, 216 and 296 in HeLa cells increased apoptosis by activation of caspase 3, while the inhibition of miR-214 had the opposite effect (Cheng et al. 2005).

Li et al. have investigated the pro-apoptotic role of miR-204 in human trabecular meshwork (HTM) cells. This miR directly targeted several anti-apoptotic genes and increased cell susceptibility to apoptosis (Li et al. 2011).

Other miRNAs have a role in the regulation of apoptosis: (1) miR-218 was found to be involved in NF-kappaB response and apoptosis induction by targeting expression of the ECOP gene (Gao et al. 2010); (2) miR-1 and miR-133 produced opposing effects on apoptosis induced by oxidative stress in rat cardiomyocytes (Xu et al. 2007). miR-1 had a pro-apoptotic function in response to oxidative stress by targeting heat shock proteins HSP60 and HSP70 and miR-133 seemed to have an anti-apoptotic role by repressing caspase 9 gene expression; (3) miR-34 family members were found to function as potent mediators of the p53-induced apoptotic pathway by targeting anti-apoptotic genes including Bcl-2, and were also found to participate in a positive feedback loop of p53 activation via increased acetylation by targeting SIRT1 deacetylase (Hermeking 2010); (4) miR-30 was shown to affect the levels of the Ubc9 and ITGB3 genes in breast tumor-initiating cells which restricted their self-renewing capacity and targeted them for apoptosis (Yu et al. 2010); (5) miR-10a was shown to participate in the TRAIL-induced apoptosis pathway leading to caspase 3 activation in human lung carcinomas (Ovcharenko et al. 2007); (6) the members of let-7 miRs family, let-7c and let-7g, were shown to target Bcl-x_L directly and Mcl-1 indirectly which led to caspase-3/7 activation and apoptosis induction in hepatocytes (Shimizu et al. 2010).

Druz et al. recently showed the up-regulation of the large miR 297–669 cluster during apoptotic conditions induced by nutrient depletion in CHO cells. One member of this cluster, miR-466h was shown to alter the expression of five anti-apopototic genes from different apoptosis-initiating pathways (bcl2l2, dad1, birc6, stat5a and smo). Antisense knockdown of miR-466h delayed apoptosis onset in nutrient-depleted conditions by decreasing caspase activation and increasing the cell viability (Druz et al. 2011). One other member of the miR 297–669 cluster, miR-669c, had been previously associated with impairments in glutathione metabolism which activated the apoptosis cascade (Lanceta et al. 2010; Maes et al. 2008).

Application of apoptosis regulation-related miRNAs in the engineering of CHO cells is possible; it should provide researchers with another tool for apoptosis inhibition. The recent sequencing of the CHO cell genome and miRNA transcriptome will provide information on gene down-regulation in CHO cells by CHO-specific apoptosis-related miRNAs (Hackl et al. 2011; Johnson et al. 2011; Xu et al. 2011). The sequencing of CHO-specific miRNAs has revealed a conserved sequence of the apoptosis-regulating miRNAs such as the miR-15/16 cluster, let-7 family, miR-214, miR-218, miR-1, and miR-10a. These miRNAs and the miR 297–669 cluster are possible targets for apoptosis pathway engineering. Due to the complexity of the PCD and the diversity of the apoptotic stimuli, it may be useful to investigate the combined effects of several miRNAs affecting genes from different stages of the apoptosis cascade, and the effects of miRNAs which seem to be involved in global regulation of the pathway. In addition, it might be worthwhile to consider the engineering of whole clusters of apoptosis-relevant miRNAs, since clustered miRNAs are known to be transcribed together as polycistronic transcripts to regulate the mRNA of genes with similar functions (Druz et al. 2011). More studies on CHO cell-specific miRNAs, their biological role and their effects on CHO cell-specific gene targets need to be conducted to suggest the most suitable miRNA candidates to be used in industrial scale bioprocesses.

5.2.3 Role of MicroRNA in Engineering of Stress Response

Stress conditions in bioreactors do not only induce apoptosis but can also affect product yield and properties (Muller et al. 2008). For example, high protein expression and limited secretion can lead to accumulation of aggregated and misfolded proteins, which can generate stress in the endoplasmic reticulum (ER). This stress was shown to be reduced by ER expansion via ectopic expression of transcriptional factor X-box-binding protein1, Xbp1 (Tigges and Fussenegger 2006), and over-expression of the protein disulfide isomerase (PDI) (Borth et al. 2005). Over-expression of ER chaperones, calreticulin and calnexin (induced by heat shock, amino acid deprivation and perturbations of Ca^{2+} levels) increased cell specific productivity of recombinant thrombopoietin in CHO cells (Chung et al. 2004; Heal and McGivan 1998).

Dissolved oxygen gradients may affect growth, metabolism, and the recombinant protein production. The gradients can be significant in bioreactors as a result of

inefficient mixing that exposes the cells to different oxygen tensions ranging from absence of oxygen (hypoxia) to above-atmospheric concentrations (hyperoxia) (Dunster et al. 1997). An example of a genetic engineering approach to reduce oxidative stress is the over-expression of the antioxidant haptoglobin which increased tolerance to oxidative stress in CHO cells (Tseng et al. 2004). The effects of shear stress on cell growth, metabolite consumption, and protein production were investigated in recombinant CHO cell cultures producing tissue plasminogen activator (tPA) and human growth hormone (hGH) (Keane et al. 2003; Senger and Karim 2003). In both cases, shear stress had a negative effect on recombinant protein production. Elevated levels of ammonia were identified as another stress condition that lowers the expression of genes managing cell cycle and protein folding, up-regulates genes affecting energy metabolism and induces protein degradation (Chen and Harcum 2006; 2007). Cold stress was shown to have positive effects on recombinant protein production in CHO cells (Fox et al. 2005) and the exogenous over-expression of the cold-inducible RNA-binding protein (CIRP) increased the titer of recombinant protein (Tan et al. 2008).

Changes in expression profiles of several miRNAs have been observed in response to ER and hypoxic stress conditions: (1) miR-708 was shown to be induced during ER stress by the transcription factor CCAAT enhancer-binding homologous protein (CHOP), and may facilitate the enhancement of ER protein-folding capacity under the stress of accelerated protein synthesis (Behrman et al. 2011); (2) miR-204 supports ER and oxidative stress induction in human trabecular meshwork cells. This miRNA inhibited two genes involved in the elimination of damaged and misfolded proteins (SERP1/RAMP4 and M6PR) and facilitated the increase of carbonylated proteins (Li et al. 2011); (3) The miR-221/222 cluster was down-regulated during ER stress in human hepatocellular carcinoma cells. The ectopic introduction of miR-221/222 mimics increased ER-stress induced apoptosis which was associated with $p27^{Kip1}$ and MEK/ERK-directed cell cycle regulation (Dai et al. 2010); (4) miR-15a, miR-16, and miRs-20a were down-regulated at hypoxic conditions in human carcinomas (Hua et al. 2006); (5) miR-26, miR-107, and miR-210 were up regulated in neoplastic cells in response to low oxygen. These miRNAs are likely to decrease the pro-apoptotic signaling in a hypoxic environment (Kulshreshtha et al. 2007). miR-210 was also found to be progressively up-regulated in endothelial cells in hypoxic conditions and inhibited receptor tyrosine-kinase ligand Ephrin-A3 which is critical in vascular development (Fasanaro et al. 2008); (6) The up-regulation of the miR-34 family, while being part of the p53 network, can be also implicated as a stress response to DNA damage, hyperactive cytokine signaling, and hypoxia (He et al. 2007); (7) The miR 17–92 cluster was shown to target hypoxia-inducible factor alpha (Hif-1α), a transcriptional factor known to regulate cellular response to hypoxia. The latter has an important role in various biological processes such as glucose metabolism, pH regulation and angiogenesis (Taguchi et al. 2008); (8) miR-31 was shown to activate Hif-1α via the inhibition of factor-inhibiting hypoxia -inducible factor (HIF) (Liu et al. 2010).

Several miRNAs were found to be associated with oxidative stress: (1) the bicistronic transcript miR-144/451 is involved in oxidative stress in erythroid cells. miR-144 was shown to modulate the oxidative stress response in K562 and primary

erythroid progenitor cells by directly affecting NRF2 gene expression which induces the expression of several antioxidant enzymes (Sangokoya et al. 2010). miR-451 protects the erythrocytes against oxidative stress and reverses differentiation defect of erythroid cells by inhibiting the intracellular regulator of cytokine signaling, 14–3-3ξ gene (Patrick et al. 2010). (2) miR-34a and miR-93 were involved in the loss of oxidative stress defense, and repress expression of genes associated with oxidative stress regulation and defense mechanism such as Sp1, Sirt1, Mgst1, and Nrf2 (Li et al. 2011).

Some miRNAs have been associated with other stress inducers in a bioreactor such as osmostic pressure, shear stresses, and nutrient depletion /gradients: (1) miR-200b and miR-717 are down-regulated by isotonic and hypertonic treatments in renal medullary epithelial cells. However, when up-regulated, these miRs inhibit the activity of transcriptional factor called osmotic response binding protein, OREBP, a major cellular osmoregulator in kidney cells and T-lymphocytes (Huang et al. 2010); (2) miR-7b was over-expressed in hyperosmolar conditions to down-regulate the protein levels of Fos. This reduces the activity of transcription factor activator protein 1 (AP1), a regulator of cellular processes, which is formed by the dimerization of Fos and Jun proteins (Lee et al. 2006); (3) miR-21 and miR-19a were induced by shear stress in endothelial cells (Qin et al. 2010; Weber et al. 2010); (4) Members of miR 297–669 cluster were up-regulated in response to nutrient depletion in CHO cells (Druz et al. 2011).

The utilization of stress-related miRNAs should be considered for bioprocess-relevant pathway engineering to eliminate negative effects on recombinant protein production. The homologs for some stress-related miRNAs (miR-7b, 93, 107, 144, 200b, 210, 708) were reported in CHO cells via next-generation sequencing (Hackl et al. 2011) and their activity can be examined in CHO cells.

5.2.4 Metabolic Disorders and Their Prevention

Accumulation of lactate and ammonia can significantly affect cell growth and production capability (Ozturk et al. 1992). Lactate is a major by-product of glucose and glutamine metabolism. When glucose is converted to lactate, glutamine metabolism supports the TCA cycle by its conversion to glutamate and later to α-ketoglutarate, which generates excessive amounts of ammonia (Muller et al. 2008). Ammonia accumulation was shown to affect cell growth and the properties of secreted protein via altered glycosylation pattern in CHO cells (Yang and Butler 2000).

The main approaches to optimize metabolic performance of mammalian cells include optimization of feeding strategies and genetic engineering of specific metabolic pathways (Irani et al. 2002). Over-expression of cytosolic pyruvate carboxylase (PC) enzyme in BHK cell cultures reduced lactate accumulation, and improved glucose and glutamine metabolism and recombinant protein production in a broad range of glucose concentrations in culture media (Elias et al. 2003; Irani et al. 2002). Knockdown of lactate dehydrogenase A enzyme (LDH-A), which catalyzes conversion

of pyruvate to lactate, reduced glycolytic flux and the specific lactate production rate in CHO cells (Kim and Lee 2007). However, despite the reduced lactate accumulation and increased rate of oxidative phosphorylation, CHO cells became more susceptible to oxidative stress after knock-down of the LDH-A (Jeong et al. 2004). Korke et al. showed that a lowered ratio of glucose consumption to lactate production in hybridoma cells culture was the result of global changes in gene expression at the transcription level (Korke et al. 2004) and may be in part due to regulation of genes by miRNAs (Muller et al. 2008).

Another tool to investigate metabolic phenotypes is a metabolomics approach coupled with ^{13}C-flux analysis of cellular metabolism and its regulation in mammalian cells. This approach offers the quantification of internal metabolic fluxes, providing researchers with comprehensive information on cellular energetics (Zamboni and Sauer 2009).

Several miRNAs have been related to metabolic control. (1) miR-375 was shown to regulate glucose homeostasis and glucose-mediated insulin secretion in pancreatic endocrine cell lines by targeting the myotrophin (Mtpn) gene (Poy et al. 2007). miR-375 was also shown to down-regulate 3′-phosphoinositide-dependent protein kinase-1 (PDK-1), resulting in decrease of insulin gene expression in primary rat islets (El Ouaamari et al. 2008). Over-expression of miR-375 decreased glucose-induced insulin secretion with no effects on glucose-stimulated ATP production or intracellular Ca^{2+} levels (Gauthier and Wollheim 2006); (2) miR-124a, miR-107, miR-30d were up-regulated, and miR-296, miR-484, miR-690 were down-regulated at high glucose conditions in pancreatic beta cells. Over-expression of miR-30d increased insulin gene expression indirectly, but had no effects on insulin secretion (Tang et al. 2009); (3) miR-122 was shown to be involved in regulation of cholesterol and lipid homeostasis in mice (Esau et al. 2006; Krutzfeldt and Stoffel 2006); (4) miR-29b involvement in amino acid metabolism was shown in HEK293 cells. This miRNA controlled the branched amino acid (BCAA) metabolism by targeting the branched-chain α-ketoacid dehydrogenase (BCKD) enzyme, known to catalyze the irreversible step in BCAA catabolism (Mersey et al. 2005); (5) Gao and colleagues showed the effect of miR-23a/b in human B lymphoma cells on the regulation of glutamine metabolism by targeting mitochondrial glutaminase (GLS) expression. It was also shown that miR-23a/b is subject to c-MYC regulation (Gao et al. 2009); (6) miR-378*, was shown to induce the metabolic shift in breast cancer cells by targeting estrogen related receptor (ERRγ), and GA-binding protein α (GABPA), two key regulators of energy metabolism. Over-expression of miR-378* reduced the activity of the TCA cycle, rendering the cells less dependent on oxidative phosphorylation and causing increased lactate production (Eichner et al. 2010).

Some metabolism-related miRNAs discussed above (miR-23a/b, -29b, -30d, -107, -122, -296, -484, -378*) were already reported in CHO cells (Hackl et al. 2011; Johnson et al. 2011). miR-dependent regulation of metabolic pathways is relatively new; therefore, comprehensive analysis of miR-mediated control of metabolic enzymes and fluxes and their effects on metabolic phenotypes, coupled with the investigation of CHO-specific miRs and gene targets, needs to be conducted.

5.2.5 *Protein Secretion Pathway Engineering*

Recombinant protein production in mammalian cells is achieved by controlling recombinant gene expression and optimizing protein secretion. Improving gene expression has received considerable attention, and approaches such as codon optimization and promoter and enhancer sequence design have been utilized (de Boer et al. 2004; Kim and Lee 1997; Masuda et al. 2000). However, limited attention has been given to protein secretion engineering (Muller et al. 2008).

One way to improve the secretion of the recombinant protein is over-expression of the spliced form of X-box binding protein1 (Xbp-1S), a transcriptional activator for a number of genes linked to protein secretion and endoplasmic reticulum (ER) biosynthesis. Over-expression of Xbp-1S in mammalian cells increased recombinant protein secretion in cases when protein accumulation exceeded the secretory capacity of the cells (Ku et al. 2008).

Kantardjieff et al. showed direct correlation between enhanced protein production and overall increase of ER and Golgi-related protein secretion system in CHO and NS0 cells at low temperature and sodium butyrate treatment (Kantardjieff et al. 2010). Therefore, the genes that are closely engaged in the secretory pathway are worth considering as potential targets for secretion pathway engineering. Suitable candidates include coat protein complexes I and II, which recruit the cargo and direct its traffic through the early secretory pathway between ER and Golgi (Muller et al. 2008; Barlowe 2000). Over-expression of Munc 18b, the regulator of the fusion of secretory vesicles with the plasma membrane, increased heterologous protein production in several mammalian cell lines (Peng 2010). In addition, the ectopic expression of the synaptosome-associated protein (SNAP-23) and vesicle-associated membrane protein 8 increased mammalian cell productivity (Peng et al. 2011).

Few miRNAs have been associated with controlling the secretory pathway in mammalian cells: (1) miR-124a and miR-96 affected the genes involved in insulin secretion in pancreatic β-cells (Lovis et al. 2008). miR-124a increased insulin exocytosis at low glucose concentrations by indirectly increasing the levels of SNARE protein SNAP25, GTPase Rab3A, and synapsin-1A and by directly targeting the GTPase Rab27A. miR-96 increased the levels of granuphilin protein, a potent inhibitor of insulin exocytosis, and decreased the expression of Rab27A-binding protein, Noc2 (Lovis et al. 2008); (2) miR-9 reduced glucose and potassium-dependent insulin exocytosis by targeting transcription factor Onecut2 (OC2) which represses Granuphilin/Slp4, that negatively affects the insulin secretion (Plaisance et al. 2006); (3) miR-375 has negative effects on the glucose-mediated insulin secretion (Gauthier and Wollheim 2006).

Although most of the knowledge about miRNA involvement in protein secretion comes from studies of pancreatic β-cells, there are some reports of miRNA connection to the senescence -associated secretory phenotype, characterized by growth arrest, increased gene expression and secretion of various proteins (Bhaumik et al. 2009). Bhamuik et al. showed up-regulation of miR-146a/b in senescent human fibroblasts. The ectopic expression of miR-146a/b was shown to target a component

of IL-1 transduction pathway and reduced the secretion of IL-6 and IL-8, thereby participating in the negative feedback loop which suppresses the imbalance of the senescence -associated secretory phenotype activity (Bhaumik et al. 2009).

The engineering of protein secretory pathway via manipulation of miRNA levels is feasible for industrial scale applications in CHO cell cultures, especially considering that homologs for miR-9 and miR-146a/b have been reported in CHO cells (Hackl et al. 2011; Johnson et al. 2011). This research is in its early stages and more studies are needed to identify the effects of manipulating specific genes on the recombinant protein secretion rates, and to identify the CHO-specific miRNAs involved in targeting those genes.

5.3 Global Modification of MicroRNA Expression to Improve CHO Cells Bioprocesses

Global modification of miRNA expression levels is another strategy to improve CHO cell performance. The fact that mature miRNA expression levels in tumors are usually lower than their levels in healthy tissues (Lu et al. 2005), and over-expression of miRNAs frequently prevents transformation of cells to cancer-like phenotype, indicates their possible anti-oncogenic role (Imam et al. 2010; Lee et al. 2010). Therefore, the global down-regulation of miRNA expression may improve proliferative capacity of the cells and eliminate the stress response activation pathways (Barron et al. 2011a).

Modification of miRNA expression levels by genetic or epigenetic means, and by varying the levels of the miRNA biosynthesis enzymes and degradation factors, should be considered for CHO cell engineering (Barron et al. 2011a). An example of genetic regulation of miRNA profiles is the effect of the oncogenic transcription factor, c-Myc, which down-regulates miRNA expression levels by direct association with promoters of pri-microRNAs (Chang et al. 2008). Another study showed the opposite effect, where c-Myc activates the pro-oncogenic miR 17–92 cluster by direct binding to this cluster's promoter (O'Donnell et al. 2005). Other transcription factors, such as Hif-1alpha, NF-κB and p53 were also shown to affect expression of miRNA clusters by interaction with their promoters (Sun et al. 2010).

Epigenetic regulation of a single miRNA or an miRNA cluster can be initiated by DNA methylation and histone modifications; it was shown by the activation of epigenetically silenced miR-127 in human cancer cell lines (Saito et al. 2006). It is also possible to alter miRNA biogenesis by knocking down the miRNA processing enzymes Dicer and Drosha, or by over-expression of degradation factors such as XRN2 (Martello et al. 2010).

The global modification of miRNA expression needs to be carefully investigated to avoid potential harmful effects on cell homeostasis. For example, even though knockout of Dicer and Drosha can be implemented to globally down-regulate miRNA expression, it can negatively affect cell-cycle progression and mitosis in some cell lines (Martello et al. 2010). More research needs to be conducted to clarify the effects of global miRNA alterations on cellular functions in CHO cells.

Table 5.1 Involvement of miRs in industrially-relevant pathways

microRNA	Reported in CHO	Affected pathway	Effect type	Target(s)
miR-1	Yes	Apoptosis	Pro-apoptotic	HSP60, HSP70
miR-1d	No	Apoptosis	Anti-apoptotic	?
miR-7	Yes	Cell growth	Growth inhibitory	EGFR
		Apoptosis	Anti-apoptotic	?
miR-7b	Yes	Osmotic stress	Inhibition of stress response	Fos
let-7	Yes	Cell growth	Growth inhibitory	c-Myc, RAS
let-7c/g	Yes	Apoptosis	Pro-apoptotic	Bcl-x_L Mcl-1
miR-9	Yes	Protein secretion	Inhibits protein secretion	OC2
miR-10a	Yes	Apoptosis	Pro-apoptotic	?
miR-15a@ miR-16	Yes	Apoptosis	Pro-apoptotic	Bcl-2
		Hypoxic stress	Possible induction of apoptosis under hypoxia	?
miR-17-5p@ miR-20a	Yes	Cell growth	Inhibit/promote growth	E2F1
		Hypoxic stress	Possible induction of apoptosis under hypoxia	Hif1-alpha
miR-18a	Yes	Cell growth	Promotes growth	?
miR-19a	Yes	Shear stress	Anti-proliferative effect under laminar shear stress	Cyclin D1
miR-21	Yes	Cell growth	Growth inhibitory Anti-apoptotic	CDC25A
		Apoptosis	Decrease in apoptosis under laminar shear stress	PDCD4
		Shear stress		PTEN
miR-23a/b	Yes	Metabolism	Control of glutamine metabolism	GLS
miR-24	Yes	Cell growth	Growth inhibitory	E2F2, c-MYC
miR-26	Yes	Hypoxic stress	Decrease pro-apoptotic signals during hypoxia	?
miR-29b	Yes	Metabolism	Control of branched amino acids metabolism	BCKD
miR-30	No	Apoptosis	Pro-apoptotic	Ubc9, ITGB3
miR-30d	Yes	Metabolism	Regulation of glucose metabolism	?
miR-31	Yes	Cell growth	Growth inhibitory	E2F2
		Hypoxia	Possible resistance to apoptosis under hypoxia	FIH
miR-34a/b/c	Yes	Cell growth	Growth inhibitory	CDK4,6, c-MYC, CREB, Notch1, E2F3
		Apoptosis	Pro-apoptotic	Bcl-2, SIRT1
		Stress response	Possible induction of apoptosis under stresses	Sp1, Sirt1, Mgst1, Nrf2
miR-93	Yes	Oxidative stress	Loss of oxidative stress defense	Sp1, Sirt1, Mgst1, Nrf2
miR-96	No	Protein secretion	Inhibits protein secretion	Noc2
miR-107	Yes	Hypoxia	Decrease pro-apoptotic signals during hypoxia	?
		Metabolism	Regulation of glucose metabolism	?
miR-122	Yes	Metabolism	Regulation of cholesterol and lipid metabolism	?

Table 5.1 (continued)

microRNA	Reported in CHO	Affected pathway	Effect type	Target(s)
miR-124a	No	Metabolism	Regulation of glucose metabolism	?
			Promotes protein secretion	Rab27A
miR-125b	Yes	Cell growth	Promotes growth	?
miR-133	No	Cell growth	Promotes growth	SRF
miR-143	Yes	Cell growth	Growth inhibitory	ERK5
miR-146a/b	Yes	Protein secretion	Restrains protein secretion	NFκB, IRAK1, TRAF6
miR-148	Yes	Apoptosis	Anti-apoptotic	?
miR-144@ miR451	Yes	Oxidative stress	Protection against oxidative stress	Nrf2
				14-3-3ξ
miR-204	Yes	Apoptosis	Anti/pro-apoptotic	Bcl2l2, BIRC2, EZR, M6PR, SERP1
		ER and oxidative stress	Stress induction	M6PR
miR-200b	Yes	Osmotic stress	Inhibition of stress response	OREBP
miR-210	Yes	Apoptosis	Anti-apoptotic	?
		Hypoxia	Decrease pro-apoptotic signals during hypoxia	Ephrin-A3
miR-214	Yes	Apoptosis	Pro-apoptotic	?
miR-216	No	Apoptosis	Anti-apoptotic	?
miR-218	Yes	Apoptosis	Pro-apoptotic	ECOP
miR-221@ miR222	Yes	Cell growth	Growth inhibitory	Kit
		ER stress	Increased ER stress-induced apoptosis	?
miR-296	Yes	Apoptosis	Anti-apoptotic	?
		Metabolism	Regulation of glucose metabolism	?
miR-372@ miR-373	No	Cell growth	Promotes growth	LATS2
miR-375	No	Metabolism	Regulation of glucose metabolism	Mtpn, PDK-1
		Protein secretion	Inhibits protein secretion	?
miR-378*	Yes	Metabolism	Reduced activity of TCA cycle	ERRγ, GABPA
miR-466h	Yes	Apoptosis	Pro-apoptotic	Bcl2l2, Dad1, BIRC6, Stat5a, Smo
miR-484	Yes	Metabolism	Regulation of glucose metabolism	?
miR-690	No	Metabolism	Regulation of glucose metabolism	?
miR-708	Yes	ER stress	Improve protein folding	RHO
miR-717	No	Osmotic stress	Inhibition of stress response	OREBP

miRs effects on industrially-relevant pathways and their respective targets. (@) indicates combines miRs with the same effect on the pathway and same targets, (?) indicates unknown miR targets in relation to specific pathways

5.4 Conclusions

In this chapter we reviewed the potential role of miRNAs in CHO cell engineering for industrial applications based on their involvement in regulation of relevant pathways in other cellular systems. Table 5.1 summarizes the effects of the reviewed miRNAs on relevant cellular pathways and targeted genes in various systems. The recent work on CHO miRNA transciptome sequencing (Hackl et al. 2011; Johnson et al. 2011) allowed detection of more than 80 % of all miRNAs shown in Table 5.1. Only a few miRNAs have been studied in CHO cells so far: miR-7, miR-21/24 and miR-466h. The potential use of miR-7 and miR-466h has already been established by the manipulation of their expression levels in CHO (Druz et al. 2011; Barron et al. 2011b). Other promising individual miRNAs for CHO cell engineering include those involved in regulation of multiple pathways: miRs 15a and 16, miRs 17–5p and 20a, miRs 221 and 222, miR-34 family, miR-21, miR-31, miR-107, miR-124a, miR-204, miR-210, miR-296, and miR-375. The availability of the CHO cell genome sequence and the creation of CHO-specific genomic and proteomic tools will help to explore the activity of these miRNAs and to identify their targets. Another approach is to re-engineer (over-express or knock down) whole miRNA clusters already reported to be involved in relevant pathways such as miR 15a/16, miR 221/222, miR 144/451, miR 17–92, and miR-34b/c clusters. Also, clusters that incorporate the individual miRs shown in Table 5.1, such as miR 297–669, miR-296/298, miR 23–27 (include miR-466h, miR-296, and miR-23a respectively) need to be considered for the generation of robust CHO cell lines.

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