# Novel adipocyte lines from brown fat: a model system for the study of differentiation, energy metabolism, and insulin action

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**Summary**

**Adipose tissue has emerged as an important endocrine regulator of glucose metabolism and energy homeostasis. By virtue of the mitochondrial protein uncoupling protein-1 (UCP-1), brown fat additionally plays a unique role in thermoregulation. Interest has focused on this tissue not only as a target for pharmacotherapy of obesity and insulin resistance but also as an endocrine tissue with leptin secretion and high insulin sensitivity. Most studies of adipocytes have been limited either to primary cell culture or to a small number of established cell lines. Recently, we have generated immortalized brown adipocyte cell lines from single newborn mice of different knockout mouse models. These cell lines retain the main characteristics of primary cells including UCP-1 expression. They display sensitive and diverse metabolic responses to insulin and adrenergic stimulation and have proven to be useful in the characterization of UCP regulation and the role of key insulin signaling elements for insulin action. Here, we outline common approaches to the generation of adipose tissue cell lines. Furthermore, we propose that the novel technique of generating brown adipocyte lines from a single newborn mouse will be instrumental in gaining further insight into the role of a broad range of signaling molecules in adipose tissue biology and in the pathogenesis of insulin resistance.**

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## Introduction

To assess mechanisms of cell function in general and, more specifically, molecular mechanisms of disease, cell lines are of pivotal significance and considered an integral part of biomedical research. In endocrine research, attention has recently focused on fat cell metabolism and function, as adipose tissue appears to be of crucial importance for the development of different components of the insulin resistance syndrome.[1,2] In this review, we will first summarize conventional techniques to create adipose cell lines as models for both white and brown fat. We will then highlight characteristics of brown adipose tissue that has attracted clinical interest for its potential therapeutic role in the treatment of insulin resistance and obesity. Finally, we present and discuss a novel brown adipocyte model system that can easily be generated from different mouse models.

## Established preadipocyte cell lines

To date, a number of different mouse adipocyte cell lines are in common use (Table 1). Four main approaches have been employed to generate these lines (Fig. 1): 1) cloning cells susceptible to adipogenic conversion by serial passage of embryonic fibroblasts, 2) treating embryonic fibroblasts with a DNA methylation inhibitor, 3) isolation of precursor cells from bone marrow, and 4) isolation and subcloning of adipogenic cells from various tumors including those from transgenic mice carrying simian virus 40 (SV40) transforming genes. A similar fifth way of cell immortalization by direct infection of proliferating primary cells with the SV40 large T antigen is part of an advanced technique to create novel adipocyte cell lines from different mouse models.

### *White adipose tissue*

3T3 L1 and 3T3-F442A cells have derived from fibroblasts isolated from disaggregated Swiss mouse embryos, immortalized by continuous passage and subcloned according to their differentiation capacity.[3–5] These cell lines can be differentiated over a period of 10 to 15 days and are considered a model for white adipose tissue although, when fully differentiated, they do not display unilocular intracellular fat stores

**Table 1.** Selected mouse adipose cell lines

| Cell lines | Adipose tissue type/origin | Reference |
|---|---|---|
| 3T3 L1/3T3 F442A | White adipose tissue/embryo fibroblasts | 3–5 |
| Ob17 | White adipose tissue/epidiymal fat cells ('de-differentiated adipocytes') | 6 |
| PFC6 | White adipose tissue/stromal-vascular fraction of epididymal fat | 7 |
| TA | White adipose tissue/embryo fibroblasts | 8–10 |
| 1246 | White adipose tissue/teratocarcinoma | 11 |
| ST13 | White adipose tissue/mammary tumor | 12 |
| BFC-1 | Brown adipose tissue/stromal vascular fraction of interscapular brown adipose tissue | 13 |
| HB2 | Brown adipose tissue/stromal vascular fraction of interscapular brown adipose tissue from p53-knock-out mouse | 14 |
| RBM-Ad, C3H10T1/2 | Brown adipose tissue/bone marrow, multipotent stem cell line | 15,16 |
| HIB 1B, T37i, and others (see text) | Brown adipose tissue/brown fat tumors ('hibernomas') from different SV40T-transgenic mice | 17–20 |

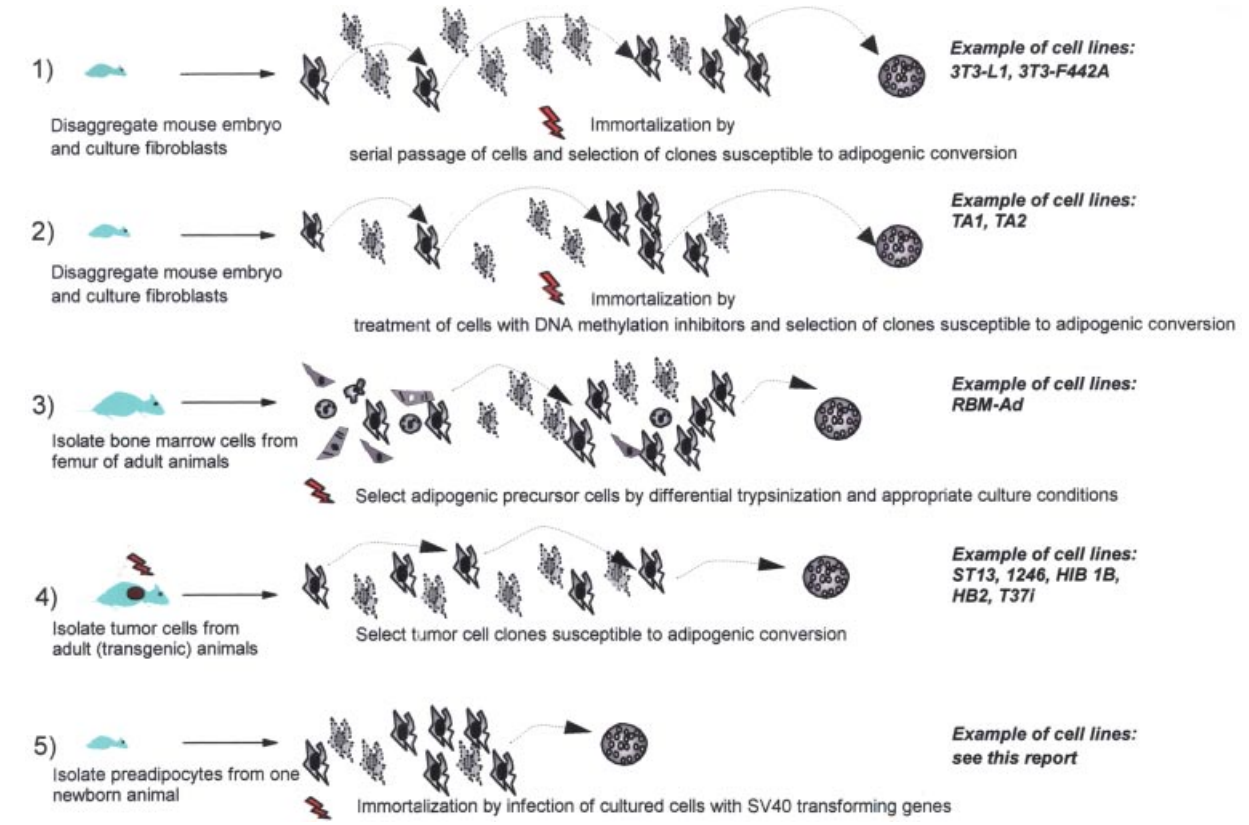
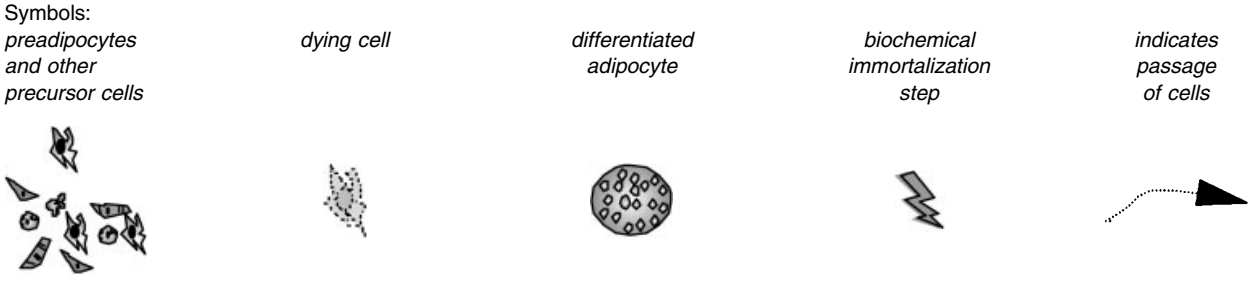



**Figure 1.** Schematic illustration of immortalization methods for the generation of adipose cell lines.

Symbols:



but multilocular fat droplets typical of brown fat cells. Serial selection has also been employed to establish Ob17 cells from epididymal fat cells of adult ob/ob mice. They most likely arise from 'de-differentiated' adipocytes.[6] An adipocyte line, derived in the same way from preadipose cells of the stromal-vascular fraction of epididymal fat, is the PFC6 line.[7]

Treatment of mouse embryo fibroblasts with 5-azacytidine, a DNA methylation inhibitor, has been shown to result in the appearance of adipogenic cells,[8] known cell lines are TA1 and TA2 cells.[9,10]

Tumors have been the origin of fat cell lines such as 1246 cells (from teratocarcinoma) and ST13 cells (from mammary tumor).[11,12]

### *Brown adipose tissue*

Cloning by serial passage has also been used to generate brown fat cell lines. The BFC-1 line has been generated from the stromal-vascular fraction of interscapular brown adipose tissue by continuous passaging and selecting clones with differentiation capacity.[13] Using the same approach, HB2 cells have been established from the knockout mouse for the tumour suppressor protein p53.[14]

From precursor cells in bone marrow, the RBM-Ad adipocyte cell line has been isolated by differential seeding. The cells morphologically and functionally resemble brown adipocytes.[15] Treatment with thiazolidinediones and insulin commits the pluripotent stem cell line C3H10T1/2 to differentiation into brown adipocytes.[16]

Furthermore, brown adipocyte lines were derived from transgenic mice carrying simian virus 40 (SV40) transforming genes. HIB 1B cells were established from brown fat tumors, hibernomas, of mice expressing SV40 genes linked to the adipocyte-specific regulatory region from the adipocyte P2 (aP2) gene.[17,18] Linking the SV40 large T antigen to the mouse urinary protein promoter[19] or the mineralocorticoid receptor[20] also resulted in brown fat tumors from which cell lines have been isolated. In this context, it should be noted that a human brown adipocyte cell line, PAZ6, has also been created by microinjection of genes encoding SV40 T and t antigens.[21,22]

All the above mentioned adipose cell lines, both from brown and white fat, have been valuable for investigating fundamental biological aspects of cell proliferation, differentiation and modes of hormonal action. To our knowledge, however, no fat cell system has been presented that allows for the rapid establishment of cell lines from a single newborn or late fetal mouse of different mouse models. In the following sections, we will first summarize some important features of brown fat to familiarize readers with the rationale for establishing brown adipocyte cell lines. We will then present and discuss an adipocyte model system that can be created from various mouse models.

## Brown adipose tissue characteristics

Brown fat is a form of adipose tissue specialized in thermoregulation.[23,24] Like white adipose tissue, it is insulin-sensitive and has endocrine functions—including secretion of leptin—but, in addition, brown fat is characterized by the expression of a tissue-specific mitochondrial protein, uncoupling protein-1 (UCP-1). UCP-1 dissipates the energy stored in the proton gradient across the inner mitochondrial membrane by mediating a proton 'leak', thereby uncoupling oxidative phosphorylation from ATP generation and producing heat instead. This process plays an important role not only for the survival of small vertebrate animals in the cold but also in the control of energy homeostasis, e. g. in mediating the increase in heat production after food intake (diet-induced thermogenesis).[24–27] In humans, brown fat is present at all ages.[25,27] However, due to its complex distribution in adults, its contribution to the control of energy balance and, potentially, the development of insulin resistance are poorly understood. Expression of UCP-1 is highly regulated by the sympathetic nervous system. In particular, activation of the β3-adrenergic receptor is closely linked to induction of UCP-1.[23,28] UCP-1 mutations, both alone and in combination with mutations in the β3-adrenergic receptor gene, have been associated with obesity in humans.[29–31] Moreover, in human and primate studies, β3-adrenergic agonists can increase insulin sensitivity and resting metabolic rate.[32,33] Thus, brown adipocytes represent an ideal model system to study regulatory elements of energy metabolism and insulin action.

## The novel brown adipocyte cell system

### *Cell isolation*

To generate adipocyte cell lines from different mouse models including those with perinatal mortality, we took the fibrostromal fraction of interscapular brown fat from one newborn or late fetal mouse (approximately 10 mg). The tissue can easily be localized and, at this developmental stage, exhibits its maximum growth. 8–10 mg of brown adipose tissue was sufficient to isolate cells that could be grown on 12-well-tissue culture plates as described elsewhere.[34]

### *Immortalization*

Immortalization was begun when cells reached 60–70% confluence and was carried out by infection of cells with the retroviral vector pBabe encoding the SV 40 T antigen. Selection was begun after 72 hours and maintained for at least two to three weeks. In general, the process from cell isolation to successful selection of immortalized cells took no longer than three to four weeks.

The principal technique of cell isolation and immortalization is based on a similar approach previously described in rat brown adipocytes.[35]

*Differentiation*

For differentiation, a procedure similar to the standard differentiation of 3T3 L1 cells was implemented. Immortalized preadipocytes were grown in culture medium supplemented with insulin and T3, and differentiation was induced for 12–48 hours with isobutylmethylxanthine, dexamethasone, and indomethacin when cells had become confluent. Full differentiation was achieved after 8–10 days. In our experience, all cell lines, established from control mice in this way, differentiated to 80–100 % as assessed microscopically, by fat-specific Oil Red O staining, and by expression of differentiation markers. Differentiation also occurred in the absence of insulin, albeit somewhat decreased and delayed. Cells could be passaged beyond 30 passages without losing their differentiation characteristics or showing cell biological signs of transformation. Routinely, cells were used between passages 10 and 20.

## Prominent features of the novel brown adipocyte lines

Figure 2 gives a diagrammatic summary of some prominent characteristics investigated so far.

*High sensitivity to adrenergic stimulation*

In non-stimulated differentiated immortalized adipocytes, UCP-1 mRNA is expressed at 70% of the level seen in brown adipose tissue control samples isolated from a mouse. Upon selective and non-selective β-adrenergic stimulation, mRNA and protein levels can be induced by 3- to 5-fold.[34]

*Insulin stimulation of the major signaling components*

Acute insulin stimulation in differentiated brown adipocytes leads to a strong increase in tyrosine phosphorylation and activation of all major insuling signaling elements including insulin receptor substrates, mitogen-activated protein kinase, phosphatidylinositol-3 kinase, and protein kinase B.[34,36–38]

*Insulin stimulation results in a robust and diverse metabolic response*

At the functional level, insulin stimulation elicits a sensitive and plurimetabolic response. Insulin-induced glucose uptake, lipogenesis, and glycogen synthesis are increased by approximately 8-fold, protein synthesis by about 2.5-fold.[39]

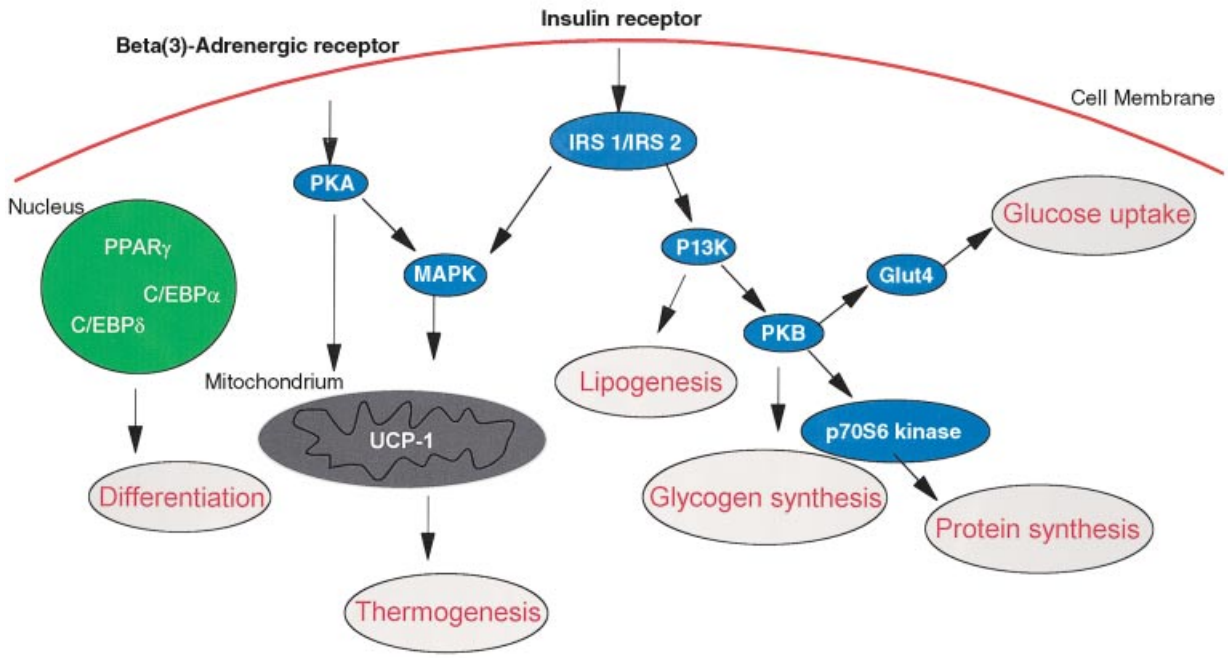



**Figure 2.** Schematic representation of important signaling pathways and functional responses in brown adipocytes generated by the novel approach. Arrows indicate investigated pathways but do not specify positive or negative influences. For molecules written in italics, knockout cell lines from respective mouse models have been created. IRS, insulin receptor substrate; PI3K, phosphatidylinositol-3-kinase; MAPK, mitogen-activated kinase; PKB, protein kinase B; PKA, protein kinase A; C/EBP, CCAAT/enhancer-binding protein; PPARγ, peroxisome proliferator-activated receptor gamma.

**Table 2.** Characteristics of brown adipocyte cell lines generated using the novel approach

| Mouse model | Differentiation | Insulin sensitivity | UCP expression |
|---|---|---|---|
| Control strains: FVB, C57/B6 | + | + | + |
| β3-adrenoceptor knockout mouse | + | + | + |
| IRS-1 knockout mouse | — | — | — |
| IRS-2 knockout mouse | + | — | + |
| IRS-3 knockout mouse | + | + | n.a. |
| PI3K knockout mouse | + | — | n.a. |

Legend: +, present or high; −, absent or decreased; n. a., not assessed

### Cell lines from mouse models of the insulin resistance syndrome

In the way described above, several brown adipocyte cell lines have been established from single newborn mice of different mouse models including knockout mice for the insulin receptor substrates 1 and 2 as well as the regulatory subunit of phosphatidylinositol-3-kinase p85α. As detailed elsewhere,[37–42] they all exhibit distinct morphological, molecular and functional characteristics that have proved useful in investigating the role of these key signaling elements for adipocyte differentiation and insulin action (Table 2).

### Prospective role of novel adipocyte lines

Studying adipocyte models has immensely promoted our knowledge of basic cellular mechanisms regulating glucose uptake and energy metabolism as well as the control of cell differentiation and proliferation. Insights gained could successfully be translated into clinical practice. One of the

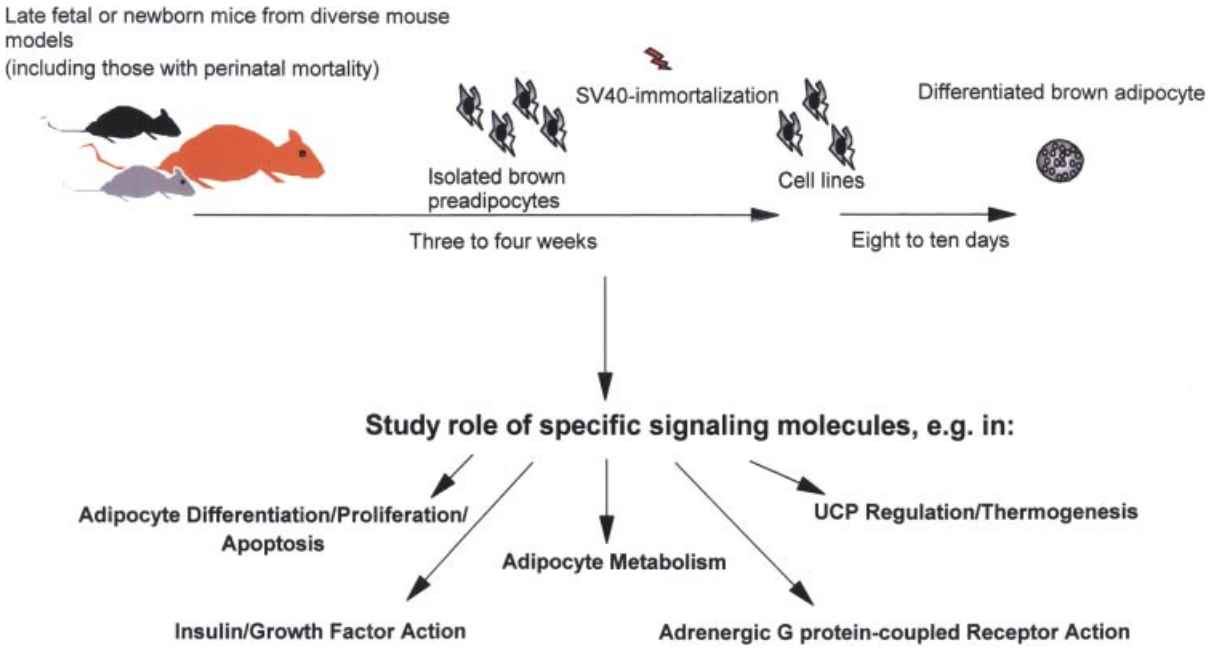



**Figure 3.** Adipocyte cell lines. Novel approaches and perspectives. Immortalization of cells isolated from transgenic mouse models by infection with SV40 transforming genes allows for combining both powerful research tools, genetic engineering of mice on the one hand, and dissecting molecular mechanisms in established cell systems on the other. The advanced technique to generate cell lines within three to four weeks from one newborn or late fetal mouse extends the spectrum of possible cell lines to mouse models with perinatal mortality. As delineated in more detail in the text, the molecular and functional characteristics of the novel cell lines promise the opportunity to study the roles of a broad variety of signaling molecules in adipocyte differentiation, proliferation, hormonal action, and energy metabolism.

examples to illustrate this progress is the use of thiazolidinediones to treat the insulin resistance syndrome. The development of this drug class hinged on research conducted in various adipocyte cell lines.

Both genetic engineering of mice and the generation of cell lines are powerful tools to better understand the biological role of many signaling molecules. The prospect of combining the methodoical repertoire of both to study the role of signaling molecules in a differentiating cell system from a broad variety of knockout mouse models, including those with perinatal mortality, promises significant molecular and functional insights. The novel adipocyte cell system is highly responsive to both, insulin and adrenergic stimulation, and can be derived within 3 to 4 weeks from a single newborn or late fetal mouse. This is an advantage over most other methods such as cloning cells from mice by serial selection, a process of immortalization whose biology is poorly understood and that takes several months. The cell system could successfully be used to investigate the role of insulin receptor substrate 1 and 2, phosphatidylinositol-3-kinase, and other signaling molecules from the respective knockout mouse models of insulin resistance.[37–42]

Brown adipocytes express all subtypes of adrenergic receptors including the β3-adrenergic receptor, a potential anti-obesity and anti-insulin resistance drug target.[28,43] The expression of UCP-1 in differentiated SV40T-immortalized brown adipocytes is sensitive to non-specific β-adrenergic as well as β3-adrenergic stimulation. The potency of β-adrenoceptor agonists to stimulate UCP-1 in the immortalized cells is comparable to primary mouse and rat brown adipocytes differentiated in culture.[28,44]

In primary rat brown adipocytes, the role of insulin and IGF signaling in adipocyte proliferation and differentiation has been studied extensively.[45–47] The kinetics of insulin-induced activation of the insulin receptor and insulin receptor substrates 1 and 2 in the immortalized brown adipocytes are similar to other systems including the widely used 3T3 L1 adipocytes.[48] Furthermore, all classical metabolic pathways are activated upon insulin stimulation including glucose uptake, protein synthesis, lipogenesis, and glycogen synthesis. The high adrenergic, notably β3-adrenergic, and insulin responsiveness of the novel cell lines has been used to analyze molecular interactions between insulin and adrenergic signaling pathways and their impact on brown adipocyte metabolism.[34,36]

From a practical viewpoint, using the technique described here, the establishment of brown adipocyte cell lines from different mouse models is a rapid and convenient process. As compared with later developmental stages, brown adipose tissue is particularly abundant in fetal and newborn mice. Homozygous and heterozygous cell lines could be derived from knockout mice for the PI 3-kinase gene p85α[39] whose homozygous offspring dies within days after birth.[49,50]

In conclusion, we propose that, in concert with existing white and brown adipocyte cell lines, the novel cell system will prove an important advance in the investigation of adipocyte biology (Fig. 3). It enables the generation of cell lines from a broad variety of knockout mouse models and will thus provide a better basis for the study of mechanisms implicated in the pathogenesis of insulin resistance and the control of energy metabolism.

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