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Activities of UDP-glucuronyltransferase, β-glucuronidase and deiodinase types I and II in hyper- and hypothyroid rats

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Abstract

We have investigated the hypothesis that uridine 5′-diphosphate (UDP)-glucuronyltransferases (UGTs) and β-glucuronidase are jointly involved in a mechanism for the storage and mobilization of iodothyronine metabolites in liver, kidney, heart and brain. Specifically, we predicted UGT activities to decrease and increase respectively, and β-glucuronidase activity to increase and decrease respectively in hypo- and hyperthyroidism. To this end we have studied the effects of thyroid status on the activities of different enzymes involved in thyroid hormone metabolism in liver, kidney, heart and brain from adult rats with experimentally induced hypo- and hyperthyroidism. We used whole organ homogenates to determine the specific enzyme activities of phenol- and androsteron-UGT, β-glucuronidase, as well as iodothyronine deiodinase types I and II. Deiodinase type I activities in liver and kidney were decreased in hypothyroid animals and, in liver only, increased in hyperthyroidism. Deiodinase type II activity was increased in hyperthyroid rat kidney only. Interestingly, in the heart, deiodinase type I-specific activity was increased fourfold, although the increase was not statistically significant. Cardiac deiodinase type I activity was detectable but not sensitive to thyroid status. Hepatic phenol-UGT as well as androsteron-UGT activities were decreased in hypothyroid rats, with specific androsteron-UGT activities two to three orders of magnitude lower than phenol-UGT activities. Both UGT isozymes were well above detection limits in heart, but appeared to be insensitive to thyroid status. In contrast, cardiac β-glucuronidase activity decreased in hypothyroid tissue, whereas the activity of this enzyme in the other organs investigated did not change significantly.

In summary, cardiac β-glucuronidase, albeit in low levels, and hepatic phenol-UGT activities were responsive only to experimental hypothyroidism. Although a high basal activity of the pleiotropic β-glucuronidase masking subtle activity changes in response to thyroid status cannot be ruled out, we conclude that hepatic, renal and cardiac UGT and β-glucuronidase activities are not regulated reciprocally with thyroid status.

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Introduction

The main secretory product of the mammalian thyroid gland is thyroxine (T\(_4\)). Deiodination of T\(_4\) to form 3,5,3′-triiodothyronine (T\(_3\)) is an important metabolic pathway, as are glucuronidation and sulfation to form glucuronidated and sulfated thyroid hormone conjugates respectively. Both conjugates are considered to be biologically inactive and have an increased water solubility, facilitating urinary and biliary excretion. In the last decade, interest in the glucuronidation of thyroid hormones has been growing because of the increased knowledge that many drugs and xenobiotics influence thyroid hormone glucuronidation (Findlay et al. 2000) and, hence, thyroid status.

We have previously shown that neonatal rat cardiac fibroblasts, in contrast to cardiomyocytes, are capable of glucuronidating thyroid hormones (van der Heide et al. 2002). This is one of few examples of extrahepatic glucuronidation of iodothyronines, and this could be relevant for local thyroid hormone metabolism in the heart. The enzymes responsible for thyroid hormone glucuronidation belong to the family of uridine 5′-diphosphate (UDP)-glucuronyltransferases (UGTs) of which many different isoforms have been identified (see review by Mackenzie et al., 1997). UGTs are not only capable of the detoxification and activation of drugs and xenobiotics (Armstrong & Cozza 2003), but are also involved in the metabolism of important endogenous substrates, e.g. bilirubin and thyroid hormones. The biochemical characterization of the different isoforms is complicated due to the broad substrate specificity (i.e. one isoform glucuronidates a wide range of substrates) and
overlapping substrate affinities (i.e. one substrate is metabolized by more than one isofrm) (de Wildt et al. 1999). Three UGTs are known to be involved in glucuronidating iodothyronines: phenol-UGT (or UGT1A9) and bilirubin-UGT (or UGT1A1), which utilize T₃ and rT₃, and androsteron-UGT (or UGT2B7), which utilizes T₄ as an acceptor for a glucuronyl residue (Visser et al. 1993, Mackenzie et al. 1997).

Glucuronidated compounds are susceptible to deconjugation by β-glucuronidase, an enzyme that is widely distributed in mammalian tissues and localized intracellularly in lysosomes and endoplasmic reticulum. Combined actions of UGTs and β-glucuronidase may play a role in futile cycling in which conjugated metabolites undergo successive cycles of synthesis to the glucuronide and hydrolysis back to the aglycone (Brunelle & Verbeeck 1993). As opposed to sulfated iodothyronine conjugates, which for example T₄ sulfate is not susceptible to deconjugation by sulfatase activity in vitro (Moë & Visser 1985), glucuronidated T₃ and glucuronidated T₄ conjugates can be deconjugated by β-glucuronidase activity to yield the native thyroid hormones (Hays & Hsu 1987, van der Heide et al. 2002). We therefore propose a functional role of conjugating and deconjugating mechanisms in the cycling of iodothyronines from a pool of stored inactivated hormones.

Based on experimental data from our group and others, and considering the observations that fibroblasts constitute two-thirds of the total cardiac cell population (Zak 1973, Tomoda et al. 2001), we suggest a role of UGT and β-glucuronidase in the storage and mobilization of iodothyronines from a pool of inactive hormones in heart. Therefore, we investigated the effects of thyroid status on the activities of a number of enzymes involved in this proposed mechanism, e.g. phenol- and androsteron-UGT and β-glucuronidase, as well as deiodinase types I and II, in heart, liver, kidney and brain from adult rats with induced hyper- and hypothyroidism.

Materials and Methods

Materials

All reagents used for isolation and cell culture were obtained from Life Technologies BV (Breda, The Netherlands), with the exception of trypsin (Boehringer, Mannheim, Germany). Polystyrene 24-well culture dishes were from Corning Costar Europe (Badhoevedorp, The Netherlands), with the exception of trypsin (Boehringer, Mannheim, Germany). Polystyrene 24-well culture dishes were from Corning Costar Europe (Badhoevedorp, The Netherlands). Iodothyronines culture dishes were from Corning Costar Europe (Boehringer, Mannheim, Germany). Polystyrene 24-well culture dishes were from Life Technologies BV (Breda, The Netherlands), with the exception of trypsin (Boehringer, Mannheim, Germany). Polystyrene 24-well culture dishes were from Corning Costar Europe (Badhoevedorp, The Netherlands), with the exception of trypsin (Boehringer, Mannheim, Germany). Polystyrene 24-well culture dishes were from Corning Costar Europe (Badhoevedorp, The Netherlands). All other chemicals were analytical grade and obtained from commercial suppliers.

Animals

Adult Wistar rats (6–10 weeks) were obtained from laboratory stock (Utrecht University, The Netherlands) and made hypo- and hyperthyroid respectively. To attain hypothyroidism, we employed an established protocol (van Hardeveld & Clausen 1984) in which rats were fed for 7 weeks an iodide-deficient diet with distilled water during the first 2 weeks and drinking water containing 144 mM KClO₄ for five consecutive weeks. This treatment has proven to result in the classic picture of hypothyroidism with decreased plasma T₄ and T₃ levels and rises in thyrotropin (TSH) (Golstein et al. 1999). As opposed to sulfated iodothyronine conjugates, of which for example T₄ sulfate is not susceptible to deconjugation by sulfatase activity in vitro (Moë & Visser 1985), glucuronidated T₃ and glucuronidated T₄ conjugates can be deconjugated by β-glucuronidase activity to yield the native thyroid hormones (Hays & Hsu 1987, van der Heide et al. 2002). We therefore propose a functional role of conjugating and deconjugating mechanisms in the cycling of iodothyronines from a pool of stored inactivated hormones.

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Samples (500 µl) of the incubation medium were taken at regular time intervals and analyzed by Sephadex LH-20 column chromatography (Mol & Visser 1985). We have previously established that the water-soluble fractions represent glucuronidated metabolites only (van der Heide et al. 2002). Fractions were analyzed for 125I activity in a γ-counter (Packard Cobra II, Packard Instruments Co., Meriden, CT, USA).

**UGT assays**

UGT enzyme assays were carried out essentially as described in Beetstra et al. (1991).

**Phenol-UGT** In short, homogenates (100 µl with a protein concentration of 1 mg/ml) were incubated with 1 mM pNP in a total volume of 200 µl incubation medium (in a final concentration of 100 mM Tris–HCl, 5 mM MgCl₂, 0·05% Brij56 at pH 7·4) in the presence or absence of 5 mM UDPGA (30 min at 37 °C). The reaction was quenched by adding 3·8 ml 0·1 M ice-cold NaOH and absorbance of the unconjugated pNP was measured at 405 nm. Specific phenol-UGT activity is expressed in mol pNP/µg per min.

**Androsteron-UGT** A 100 µl homogenate suspension, with a protein concentration of 1 mg/ml, was incubated with 100 µM androsteron using [3H]androsteron as tracer, in a total volume of 200 µl incubation medium (100 mM Tris–HCl, 5 mM MgCl₂, 0·05% Brij56 at pH 7·4) in the presence or absence of 5 mM UDPGA (30 min at 37 °C). The reaction was quenched by adding 2 ml ice-cold distilled water. To separate the water-soluble phase from the water-insoluble phase 2 ml ethyl acetate was added and mixed thoroughly. Samples were centrifuged for 10 min at 150 g and the ethyl acetate phase was removed. These steps were repeated once with fresh ethyl acetate, after which the water phase, containing conjugated androsteron, was analyzed for 3H activity in a β-counter (Packard Cobra II, Packard Instruments Co.). Specific androsteron-UGT activity is expressed as mol androsteron/µg per min.

**β-Glucuronidase**

β-Glucuronidase activity was assayed according to Fishman (1974). In short, 100 µl homogenate, with a protein concentration of 1 mg/ml, was incubated with 1 mM phenolphthalein–β-glucuronide in a total volume of 250 µl incubation medium (70 mM acetic acid at pH 5·0) for 60 min at 37 °C. Incubations without protein were used as a negative control; a commercial purified β-glucuronidase preparation was used as a positive control. Phenolphthalein served as a standard. The reaction was quenched by adding 250 µl ice-cold 5% trichloroacetic acid (TCA). Samples were neutralized by adding 500 µl glycine at pH 12·9. Absorbance was measured at 540 nm. Specific β-glucuronidase–UGT activity was defined as mol phenolphthalein/µg per min.

**Deiodinases**

Assays for deiodinase activities were performed essentially as described by Visser et al. (1988).

**Deiodinase type I** Homogenates (10 µg) were incubated for 15 min at 37 °C with 1 µM rT3 and tracer ([125I]rT3, 10⁶ c.p.m./ml) in 200 µl incubation medium (100 mM phosphate buffer, 2 mM EDTA, 10 mM DTT at pH 7·2) in the presence or absence of 100 µM PTU. The reaction was quenched by adding 100 µl 5% ice-cold BSA followed by 500 µl 10% TCA for deproteinization. Samples were centrifuged (15 min at 4 °C, 1500 g) and 500 µl of the supernatant was acidified with 500 µl 1·0 M HCl. Liberated iodide was analyzed by Sephadex LH-20 column chromatography, collecting the first three 1 ml 0·1 M HCl eluates. A crude liver homogenate of an untreated animal served as a positive control. The specific deiodinase type I activity was thus measured as the PTU-sensitive outer ring deiodination of rT₃.

**Deiodinase type II** The methods were the same as in our deiodinase type I assays, except for using 0·5 µM T₄ as a substrate and 20 mM DTT. PTU was omitted from the incubation medium.

**Ouabain binding assay**

The Na⁺/K⁺-ATPase concentration in soleus muscle was quantified by measuring [3H]ouabain binding capacity in the presence of vanadate (VO₄) (Nørgaard et al. 1983). Thawed samples (4–8 mg) were pre-washed twice for 10 min at 37 °C in unlabeled buffer solution (24 mM Tris, 3 mM MgSO₄, 1 mM VO₄, 250 mM sucrose, pH 7·3) to remove any Na⁺ and K⁺ present, so as to avoid interference with the binding of ouabain and/or vanadate. Samples were incubated (37 °C, 120 min) in a buffer containing 2·2 MBq/ml [3H]ouabain and 1 µM unlabeled ouabain. Unspecific binding was assessed by incubating a set of muscle samples in the same buffer but with 1 mM unlabeled ouabain. Unbound [3H]ouabain was removed with four 30-min washing steps with ice-cold unlabeled buffer solution. Each sample was then weighed and put into a counting vial, to which 0·5 ml 5% TCA containing 0·1 mM ouabain was added. Finally, after a 16-h extraction period (4 °C), 3 ml scintillation cocktail (Optiphor) was added and the ³H activity was measured by liquid scintillation counting (Minaxi Tri-carb, 4000 series; Packard Bioscience BV, Groningen, The Netherlands). Specific binding capacity was defined as mol [³H]ouabain per g wet weight.
Statistics

All data are shown as means ± S.E.M. unless indicated otherwise. Statistical significance was evaluated by Student’s t-test or Welch’s alternate t-test where appropriate. Statistical significance was accepted at P ≤ 0.05. The number of observations (n) is shown in parentheses.

Results

Verifying thyroid status

Table 1 shows that the relative weights of heart and kidneys were significantly increased in hyperthyroid rats, and that the relative weights of heart and liver were significantly decreased in hypothyroid rats. During dissection it was evident that hyperthyroid organs were much more richly innervated by blood vessels than their controls and hypothyroid organs less (data not shown). It is well established from studies in rat and human skeletal muscle that ouabain binding site density reflects the total population of functional Na⁺/K⁺ pumps (Clausen 1998), which changes proportionally to thyroid status (Kjeldsen et al. 1984, 1986, Everts 1996). Table 2 shows that soleus muscle from hyperthyroid rats expressed significantly more ouabain binding sites than their controls, whereas hypothyroid rats expressed fewer binding sites. The results show that the applied treatments efficiently induced hyper- and hypothyroidism in our experimental animals.

Deiodinase types I and II

In agreement with observations reviewed by Köhrle (1999), we found that deiodinase type I activity in control animals was more than tenfold higher in liver and kidney than deiodinase type II activity (compare Fig. 1a and b). In control heart and brain, however, the activities of deiodinase type I were of the same order of magnitude, i.e. around 6 fmol/µg per min. Cardiac and cephalic deiodinase type II activities were also of the same order of magnitude, i.e. around 0.3 fmol/µg per min.

Figure 1a further shows that deiodinase type I activities in liver and kidneys paralleled thyroid status (i.e. increased in hyperthyroidism, although not statistically significant for hyperthyroid kidney, and decreased in hypothyroidism), a result that is in line with other studies (Köhrle et al. 1991). In heart and brain, deiodinase type I activities were unaffected by thyroid status. Deiodinase type II activities were significantly increased in hyperthyroid kidney. In hypothyroid liver and kidney, deiodinase type II only displayed a trend towards decreased specific activities (Fig. 1b).

Effect of PCP

One micromolar PCP inhibits glucuronidation of T₄ and T₃ by neonatal cardiac fibroblasts, as measured by the appearance of water-soluble glucuronated iodothyronine metabolites by 89 and 75% respectively (Fig. 2).

UGT1

Phenol-UGT Phenol-UGT activities did not respond to a hyperthyroid status (Fig. 3). Hepatic and renal
phenol-UGT activities were decreased in hypothyroidism, being significant only in liver ($P < 0.05$). In heart, a specific activity of 2–4 pmol/µg per min was found, which did not change significantly with the thyroid status of the animal.

**Androsteron-UGT** In Table 3, hepatic androsteron-UGT activities are shown. In hyper- as well as hypothyroid liver homogenates, specific activities were decreased, being significant only in hypothyroid liver ($P < 0.05$). In kidneys and brain, activities were around 100-fold lower and it was therefore not possible to obtain reliable estimates of androsteron-UGT activity in these organs. In the heart, androsteron-UGT activity was about tenfold lower than in liver, but well above detection limits and was not influenced by thyroid status (data not shown).

**β-Glucuronidase**

All organ homogenates contained β-glucuronidase activities. The lowest activity levels were measured in the heart, which were decreased by 77% in hypothyroid animals (Fig. 4). No effects of experimental hypo- or hyperthyroidism on specific β-glucuronidase activities were observed in liver, kidneys and brain.

**Discussion**

In many studies different UGT activities have been measured, but most of these involved toxic xenobiotics and were performed in liver. This study investigated the involvement of hepatic as well as non-hepatic UGT and β-glucuronidase activities in the metabolism of endogenous substrates, e.g. thyroid hormones, and their putative

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Figure 1 (a) Specific deiodinase type I (D1) activities in whole organ homogenates of hyper- and hypothyroid rats ($n=4$; hypothyroid brain, $n=2$; hypothyroid kidney, $n=8$; means ± S.E.M., *$P < 0.05$ and **$P < 0.01$ with Welch’s alternate $t$-test). Please note the different scales of the primary and secondary $y$-axes.

(b) Specific deiodinase type II (D2) activities in whole organ homogenates of hyper- and hypothyroid rats ($n=4$; hypothyroid brain and kidney, $n=2$; means ± S.E.M., **$P < 0.01$ with Student’s $t$-test). ctrl hyper, hyperthyroid control; hyper, hyperthyroid; ctrl hypo, hypothyroid control; hypo, hypothyroid.

Figure 2 Production of thyroid hormone (TH) glucuronides by cardiac fibroblasts of neonatal rats exposed to 1 µM PCP, at $t=48$ h (T$_4$G control, $n=6$; T$_3$G control, $n=7$; with PCP, $n=8$; means ± S.E.M., **$P < 0.01$ with Welch’s alternate $t$-test). T$_4$G, T$_4$-glucuronide; T$_3$G, T$_3$-glucuronide.

Figure 3 Specific phenol-UGT activities in whole organ homogenates of hyper- and hypothyroid rats ($n=4$, ND = not detectable, means ± S.E.M., *$P < 0.05$ with Welch’s alternate $t$-test). ctrl hyper, hyperthyroid control; hyper, hyperthyroid; ctrl hypo, hypothyroid control; hypo, hypothyroid.

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role in cycling thyroid hormones in and from a pool of biologically inactive conjugated iodothyronines. Liver and kidneys play a stabilizing role in circulating thyroid hormone levels (Köhle et al. 1991, Hennemann et al. 2001) and we have put forward the hypothesis that cardiac fibroblasts play a stabilizing role as such in heart tissue (van der Heide et al. 2002). Because glucuronidation is generally accepted to be a clearance mechanism, we would predict increased and decreased UGT activities in hyper- and hypothyroid organs respectively. Because β-glucuronidase has the capacity to deconjugate glucuronidated iodothyronines, this enzyme potentially plays a role in mobilizing re-activated thyroid hormones from a pool of inactive iodothyronines. Therefore, we predicted reciprocal effects on β-glucuronidase activity with respect to UGT activities in response to changing thyroid status.

From the present study, it appears that mainly hepatic phenol-UGT (UGT1A9) activity is sensitive to hypothyroidism. This result is partly in line with our hypothesis, which predicts that with low circulating T₃ levels a reduced activity would be appropriate to increase the bioavailability of thyroid hormone. Parallel with phenol-UGT, androsteron-UGT (UGT2B7) activity, mainly in liver, is also sensitive to hypothyroidism, although the specific enzyme activity is several orders of magnitude lower than that of phenol-UGT. Measured at the gene expression level, primarily UGT1 isoforms were found to be differentially influenced by iodothyronine levels: in vivo T₃ treatment affected mRNA levels of UGT1 isoforms in primary cultures of rat hepatocytes, resulting in an increase of UGT1A6, and decreases of UGT1A1 and UGT1A5 mRNA levels. Isozymes of the UGT2 family, e.g. UGT2B1 and UGT2B3, were not sensitive to T₃ (Masmoudi et al. 1996, 1997a,b, Li et al. 1999). Our results obtained on UGT1A1 agree with these observations. Furthermore, we found that androsteron-UGT, a member of the UGT2 family, is sensitive to hypothyroidism.

In heart, phenol-UGT as well as androsteron-UGT activities were present, albeit not responsive to thyroid status. Both hepatic UGT activities were sensitive to hypothyroidism, as the activity of this enzyme activity was decreased in hypothyroidism. Apparently, the responsiveness of these UGT1 isoforms is organ specific. The glucuronidating capacity of the liver is probably an expression of its role in the biliary excretion of conjugated exogenous and endogenous substrates.

The specific phenol-UGT activities we measured in liver and heart of adult rats were in the same order of magnitude of those measured in neonatal rat tissues in our previous study (van der Heide et al. 2002). In whole liver homogenate, specific activities were higher in adult than in neonatal tissue, whereas in whole heart homogenate activities were lower. These results could indicate that hepatic glucuronidation of iodothyronines is relatively more important in adult life, whereas cardiac glucuronidation of thyroid hormones is more important in the neonatal period. This hypothesis should be investigated in specifically designed experiments.

Interestingly, we found that β-glucuronidase activity is sensitive to hypothyroidism only in heart. There is, however, no reciprocal correlation with the UGT activities. Both UGTs as well as β-glucuronidase activities decrease in hypothyroidism. UGTs and β-glucuronidase apparently do not jointly participate in a cycle of de-activating and re-activating of thyroid hormones. However, β-glucuronidase is a pleiotropic enzyme (Fishman 1974), and it is possible that a high basal activity masks subtle changes in enzyme activity related to thyroid hormone metabolism.

Hays & Hsu (1988) reported that T₄ glucuronides have lower affinities for plasma proteins than unconjugated T₄. In addition, Hays & Cavalieri (1992) found that T₄ glucuronide is a substrate for deiodinase type I, resulting in T₃ glucuronide, which is subsequently deconjugated to form T₃. Through this metabolic pathway, the glucuronidation of T₄ might represent a mechanism of fine-tuning the thyroid hormone level by keeping a pool of prohormone available for deiodination.

PCP is a known inhibitor of a number of different enzymes (Igisu et al. 1993, Moorthy & Randerath 1996,

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<th>Table 3 Androsteron-UGT activity in liver homogenates of hyper- and hypothyroid rats and their respective controls (pmol/µg per min, P value with respect to controls, n=4)</th>
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Figure 4 Specific β-glucuronidase activities in whole organ homogenates of hyper- and hypothyroid rats (n=4; hypothyroid brain, n=2; means ± S.E.M. *P≤0·05 with Student’s t-test). ctrl hyper, hyperthyroid control; hyper, hyperthyroid; ctrl hypo, hypothyroid control; hypo, hypothyroid.
Kester et al. 2000), and it has also been used for selective inhibition of sulfation (Mulder 1986). However, from the inhibitory action of PCP on the glucuronidating capacity of fibroblasts and, hence, UGT activity, we conclude that PCP cannot be used to discriminate between water-soluble or glucuronidated thyroid hormone metabolites. This is supported by results obtained by others, who report that PCP inhibits sulfation as well as glucuronidation of 1-naphthol and iodothyronines (Schuur et al. 1999, Bostrom et al. 2000).

UGTs are not only involved in conjugating and clearing exogenous substrates, but also in thyroid hormone metabolism. These different roles of UGTs in the mammalian body are exemplified by the observation that xenobiotics and T₃ differentially regulate bilirubin UGT in rat liver microsomes (Goudonnet et al. 1990). The effects of different exogenous substrates were found to be additive (Jennitz et al. 2000), indicating different pathways for the induction of UGTs. This is in line with the observation of Masmoudi and co-workers (1997a) who found that the transcriptional control of the UGT1A1 gene required de novo protein synthesis, whereas the activation induced by 3-methylcholanthrene did not. Results from these studies suggest that different UGT induction pathways are coupled to distinctive roles of UGT isozymes, e.g. xenobiotics clearance and thyroid hormone metabolism.

In summary, cardiac β-glucuronidase, albeit in low levels, and hepatic UGT activities were responsive only to experimental hypothyroidism. Although a high basal activity of the pleiotropic β-glucuronidase masking subtle activity changes in response to thyroid status cannot be ruled out, we conclude that hepatic, renal and cardiac UGT and β-glucuronidase activities are not regulated reciprocally with thyroid status.

We are currently investigating possible physiological effects of glucuronidated iodothyronines on cardiomyocyte cell function, employing the rat cardiac myoblast cell line H9c2(2–1) and monitoring cell proliferation and differentiation, and the expression of appropriate protein markers (e.g. MHC isoforms and SERCA).

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